INDEPENDENT REGULATION OF ALANINE AND ARGININE TRANSPORT IN HUMAN INTESTINAL EPITHELIAL CELL LINE CACO-2

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1993

This dissertation is dedicated to my wife Jun, and my parents.

ACKNOWLEDGEMENTS

I would like to thank Dr. Bruce Stevens, chairman of my supervisory committee, for his tremendous support in both my professional and personal life. Words can hardly express my deep appreciation. Working under his supervision has been a wonderful experience.

I would also like to thank members of my supervisory committee, Dr. Edward Copeland, Dr. George Gerencser, Dr. Michael Kilberg, and Dr. Souba for their support and valuable suggestions.

I would like to express my appreciation to Dr. Colin Sumners for providing the cell culture equipment through out my study. I would also like to thank Ms. Tammy Gault, for teaching me cell culture technique and sharing the cell culture equipment.

I would like to take this opportunity to thank all the faculty members of the Physiology Department for making graduate study an enjoyable experience.

Finally, I specially thank my wife and my family for their unconditional support and great family value.

TABLE OF CONTENTS

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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May 1993

Chairperson: Bruce R. Stevens
Major Department: Physiology

Membrane transporter systems serving arginine (Na^+-independent system y^+) and alanine (Na^+-dependent system B) were investigated in the human intestinal Caco-2 cell line. The uptake kinetics were different for each transport system. For each system, the V_{max} was greater in undifferentiated cells compared to differentiated cells, while the K_m values were each unaffected by cell differentiation status. Amino acid substrates unique to System y^+ acutely stimulated only system y^+ activity, while substrates unique to system B only stimulated system B activity. For each transport system, the ranking of amino acid stimulation was directly correlated with the degree of competitive analogue inhibition (assessed by Dixon analysis). The prolonged substrate induction of system B activity, but not system y^+ activity, was prevented by the protein synthesis inhibitor cycloheximide. Peptide growth

factors epidermal growth factor (EGF) and transforming growth factor-alpha (TGF∝) each stimulated system B and system y^+ activity following a lag period of several hours. EGF/TGF∝ activation was abolished by cycloheximide, or by inhibitors of protein kinase C. Phorbol esters stimulated system B and system y^+ activity following a lag period of several hours, and this stimulation was prevented by cycloheximide and inhibitors of protein kinase C. For each transport system, EGF, TGF∝, and phorbol ester increased the V_{max} but not the K_m. Together these data suggest that (1) Caco-2 epithelial differentiation status is associated with regulation of amino acid transport; (2) amino acid transporter system B and system y^+ are regulated independently; (3) amino acid substrates up-regulate their own transporter's activity via trans-stimulation or by a mechanism involving de novo protein synthesis; (4) EGF and TGF∝ likely activated protein kinase C in the up-regulation of system B and system y^+ activity via a de novo protein synthesis mechanism.

CHAPTER 1
INTRODUCTION

1.1 Introduction

Amino acid transport by the small intestine is a vital process involved in whole-body nitrogen balance. Intestinal absorption studies have received considerable attention during the past 35 years, primarily focussing on description of the site of absorption within the mucosa, classifications of uptake pathways based on substrate selectivity, and phenomenological kinetic mechanisms of transporter function using the universal principles of membrane transport (Hopfer, 1987; Munck, 1981; Stevens et al., 1984, Stevens, 1992a,b). Past and current studies were conducted in a variety of species and at various stages of development. These studies have included patients and intact animals with inborn errors of transport (Desjeux et al., 1980), perfused intestines, isolated membranes from the brush border and basolateral surfaces (Stevens et al., 1982, 1984; Mircheff et al, 1879, 1980), and enterocytes freshly isolated from mucosa (Reiser and Christiansen, 1971a,b,c), or grown as in vitro cell lines (Pan et al., 1991).

This project concentrates on amino acid absorption from the outer environment; the role of the intestine in inter-

organ amino acid flow is beyond the scope of this project. The small intestine is unique in extracting amino acids, in contrast to other internal organs. The enterocyte amino acids transport systems, especially those at the brush border membrane are unique in substrate and modes of regulation. Unlike the amino acid transport substrate adaptive down-regulation universally observed in many internal organs, substrates in the small intestine up-regulate their transporter activities in vivo studies (Diamond, 1991; Stevens, 1992a,b).

1.2 Amino Acid Absorption in the Small Intestine

Like the membrane transport processes in internal organs, small intestinal amino acid transport has been studied qualitatively and quantitatively based on uptake phenomena and non-equilibrium thermodynamic principles.

The small intestinal mucosa separates the outer environment from the internal milieu. The intestinal mucosa is composed of a single layer of historically polarized epithelial cells (enterocytes) which are joined by a tight junction to form a continuous layer. Amino acid transport across the mucosa is mainly a trans-cellular phenomenon. The epithelial villous cells are responsible for amino acid absorption (Munck, 1981; Wilson, 1962). Along the crypt-villus axis the enterocytes originate from stem cells within the crypt. The undifferentiated immature cells rapidly proliferate

and migrate up the villi to become the mature villous cells. The well-developed enterocytes stay at the villus tip for several days, and then are shed away to the intestinal lumen. The location of greatest individual amino acid absorption differs among species along the oral-aboral axis of the small intestine (Diamond, 1991; Stevens, 1992a,b). The lumen to blood absorption involves the movement of amino acid through a series of aqueous and membrane compartments (Hopfer, 1987; Stevens, 1992a,b). Each compartment acts as a barrier which influences the overall amino acid movement across all the compartments. The brush border apical membrane of these enterocytes is the initial active step regulating the flow of amino acids from the intestinal lumen into the cell cytosol.

1.2.1 Lumen to Blood Amino Acid Movement

Beginning in the lumen, amino acids travel through an unstirred water layer, the apical membrane, the cytosol, the basolateral membrane compartments, and finally capillary endothelium. Each compartment determines the "real" amino acid concentration reaching the following compartment.

The unstirred water layer is about 50 μm thick layer and is composed of water and mucous/glycocalyx. Amino acids diffuse across the unstirred water layer. The amino acid that diffuses across the unstirred layer then reach the apical or brush border membrane. This plasma membrane is a bilayer phospholipid structure separating the cell cytosol environment

from the lumen. Amino acids cross surface by a simple passive diffusion plus some carrier-mediated transport mechanisms. In some instances, the amino acid is transported via a secondary active transport mechanism against its own electrochemical gradient across the membrane. The accumulated amino acids in the cytosol then exit via passive diffusion or/and carrier-mediated transport mechanism at the basolateral membranes. Each of the barriers can influence the rate of the amino acid absorption: the "true concentration" of the amino acids reaching the brush border membrane is determined by the amount of substrate within the unstirred water layer, rather than the bulk phase concentration present in the lumen. The brush border membrane and the basolateral membrane possess biochemically and histologically different structures. Some of the amino acid transport systems at the brush border membrane are not found in the basolateral membrane and the other internal organs (Stevens, 1992a,b). On the other hand, the basolateral membrane possesses many of the same transporters as the membrane found at other internal organs.

1.2.2 Modes of the Amino Acid Transport in the Small Intestine

The membrane amino acid transport movement is classified into two general categories: simple passive diffusion and carrier-mediated transport. The carrier-mediated transport is further divided to Na^+-independent facilitated transport and Na^+-dependent secondary active transport

mechanisms.

In the case of the simple passive diffusion, the membrane electrochemical gradients and permeability coefficients of the amino acid govern the direction and rate of the passive amino acid movement across the membrane.

In the case of the Na^+-independent carrier-mediated transport mechanism, amino acid is carried by its specific transporter across the membrane, directed by the electrochemical acting across the membrane. In the case of charged amino acids such as arginine, the negative membrane potential can drive it against its chemical gradient.

In the case of the secondary active transport, a series of spatially separated events occur that couple energy derived from ATP hydrolysis to solute flux. The Na^+/K^+-ATPase at the basolateral membrane creates electrochemical Na^+ and K^+ gradients across the basolateral and the apical membranes. The Na^+/amino acid cotransporters at the apical membrane bind to amino acids, and utilize this Na^+ electrochemical energy ($\Delta\mu_{Na}$) for concentrative uptake of amino acids across the apical membrane. The accumulated amino acids inside the enterocytes exit via the Na^+-independent facilitated transporters and the passive diffusion at the basolateral membranes.

A kinetic model of Na/amino acid transport describing the secondary active transport has been recently examined Stevens (Stevens, 1992a) as a paradigm for all Na^+-dependent systems.

This model describes an prefer-ordered mechanism with the Na^+ activator ion binding preferentially first to the cis transporter conformation, and this binding increases the affinity for amino acid binding to the cis side of the transporter. Amino acid then binds to transporter. The cis-complex isomerizes to place Na^+ and amino acid on the trans side, and the substrates are released to the cytoplasm by either a random or ordered sequence. The trans transporter conformation isomerizes back to cis-transporter conformation. The overall rate-limiting step is the isomerization of the two transporter forms (cis and trans). In the absence of trans amino acid, the influx is predicted by: (Stevens & Wright, 1987): $J^A = (J^A_{max} - [A])/K_m + [A]$, where J^A = amino acid flux, J^A_{max} = maximal flux rate, and the apparent affinity K_m for solute A is a function of both K_{Na} (the apparent dissociation constant for dissociation and binding):

$$K_m = ((K_{Na}/[Na])^n + (nK_{Na}/[Na]) + 1)\ K_A$$

where K_A is the apparent amino acid-carrier dissociation constant, and n is the Hill coefficient describing the number of Na^+ ions coupled to movement of each amino acid molecule. Note that regulation of the transporter activity could conceivably occur by modifying J_{max} (i.e, activity of the functional transporter), or apparent K_m (which includes the Na^+ affinity and substrate modulation).

1.2.3 The Amino Acid Transport Systems in the Small Intestinal Membrane

Christensen and colleagues developed the original criteria to discriminate different amino acid transport systems in mammalian cells (Christensen, 1975, 1984, 1985 & 1990) through substrate specificity, ion-dependency, transport kinetics, and numerous other characteristics. Many facilitated and Na^+-dependent secondary active transport systems such as Systems A, ASC, L and y^+ which were first described in non-epithelial cell were found in many cell types including intestinal epithelial cells (Kilberg et al., 1993; Stevens, 1992a).

Much work has been conducted in the amino acid transport system classification at the tissue, cellular, and membrane vesicle levels in the intestine (Munck, 1981; Stevens et al., 1984; Hopfer, 1987; Stevens, 1992a,b). The major tools for the membrane transport system classification are (1) substrate preference; (2) ion-dependency; (3) substrate uptake kinetics, (4) patterns of analogue cross inhibition of amino acids, and (5) specific renal and intestinal inborn amino acid malabsorption syndromes (Stevens et al., 1984; Wright et al., 1986, Kilberg et al, 1993). Certain amino acids transported by a single transporter are used to test for the presence of the characteristic transporter. For example, α-methylaminoisobutyric acid (MeAIB) and pipecolic acid are thought to be transported only through the System A and the System IMINO, respectively (Christensen, 1975; Stevens & Wright 1985, 1987; Wright 1985). Due to variations among the

animal species, the stages of development, the tissue studied, and the methods used in amino acid transport systems studies, many different systems have been reported in the small intestine (Munck, 1981; Stevens et al., 1984; Hopfer, 1987; Stevens, 1992a,b). With some conflicts, there is a similarity in the amino acid transport systems among different species. The functionally and biochemically distinct brush border membrane and basolateral membrane possess different transport systems. The compiled membrane transport systems at the brush border membrane and basolateral membrane will be discussed individually.

Several distinctive transport systems are found only at the brush border membrane (Kilberg et al., 1993; Stevens et al., 1982, 1984, Stevens, 1992a,b). One of these is System B. System B is described as a strictly Na^+-dependent system broadly selective for the dipolar (neutral) amino acid alanine, serine, cysteine, glutamine, and interacts with 2-amino-2-norbornanecar-boxylic acid (BCH) and threonine. At first it was named System NBB "Neutral Brush Border" (Stevens et al, 1982, 1984; Stevens, 1992a,b). System B is characteristically similar to System $B^{0,+}$ described in blastocyte (Van Winkle et al., 1985). Both System B and System $B^{0,+}$ broadly transport dipolar amino acids, except that System $B^{0,+}$ interacts with cationic amino acids, while System B is not interactive with cationic amino acids. System B has been reported to exist in the apical membrane of rabbit (Stevens et

al, 1984), pig (Maenz et al., 1992), dog (Bulus, 1989), human fetal (Malo, 1991), lower vertebrate small intestines (Ahearn et al., 1991), and in the undifferentiated and differentiated enterocytic Caco-2 cells (Pan et al, 1991; Souba et al., 1992). Another distinct amino acid transport system found only at the intestinal apical membrane is System IMINO (Stevens & Wright, 1985, 1987; Wright et al, 1985; Stevens, 1992a,b). System IMINO is also a strictly Na^+-dependent system highly selective to heterocyclic imino acid such as proline and pipecolate. System IMINO uptake has been reported in many species intestine (Ahearn et al., 1991; Karasov et al., 1986, 1987; Moe et al., 1987; Munck, 1983; Stevens et al., 1984, 1992a,b).

Na^+-dependent transport Systems A and ASC (Christensen et al., 1965; Oxender et al., 1963; Kilberg et al., 1981, 1993), serve dipolar amino acid in the non-epithelial cell membrane, and reportedly exist in the guinea pig apical membrane (Del Castillo & Muniz, 1991; Hayashi et al., 1980). But up to date, no definite test has been able to discriminate them from the System B or System IMINO. Other Na^+-dependent systems, X_{AG}^- serving D-aspartate and glutamate, β serving β-alanine and taurine were also reported in the intestinal apical membrane (Hofper, 1987; Munck, 1990, 1992; Miyamoto et al., 1990a,b). Some studies also suggested possible existence of a System N for glutamine transport (Salloum et al., 1990, 1991).

There are three Na-independent transport systems at the

intestinal apical membrane. One is System L (Christensen et al, 1963, 1969, 1975) which transports large neutral amino acids and favors lipophilic substrates such as phenylalanine, leucine, and BCH. System L excludes β-alanine. A second is System y^+ (Christensen, 1964, 1966) which prefers cationic amino acids such as lysine and arginine, although it tolerates the substrate combination of sodium plus neutral amino acids such as homoserine. A third is System $b^{0,+}$ (Van Winkle et al, 1985, 1987, and 1988) which serves neutral amino acids and cationic amino acid, and interacts with BCH.

Recent cloning of cDNA encoding System y^+ (Kim et al., 1991; Wang et al., 1991) provides a breakthrough in the membrane transport systems studies. It is possible to study membrane transport systems using the traditional phenomenological method as well as more advanced molecular biological methods. The finding of the same protein serving as both the System y^+ transporter and a retrovirus receptor not only make the possible molecular studies of membrane amino acid transporters, but the physiological or pathological relationships among the nutrient absorption and cell functions in healthy and disease states. Recent cloning of cDNAs NAA/D2, rBAT, and F4 for putative regulatory protein for Systems $b^{0,+}$, y^+ or $B^{0,+}$ (Betran et al., 1992; Magagnin et al, 1992; Tate et al., 1992; Wells et al., 1992a,b) were also reported.

All amino acids passively diffuse across the apical membrane with their permeability rates directly proportioned

to their hydrophobicity. The order of permeability diffusion coefficients is phenylalanine > β-alanine > mannitol > alanine > MeAIB > proline > glycine > lysine (Stevens et al., 1982,1984). At high luminal amino acid concentration, the passive diffusion may be the predominant transport ways in the intestine. The carrier-mediated transport systems may be the favored route at lower concentrations.

In contrast to the apical membrane, all the basolateral membrane amino acid transport systems studied also exist in other non-epithelial membrane. These include Systems ASC and A, and Na^+-independent Systems y^+ and L, plus simple passive diffusion. The characteristics of these systems are as the same as those described in the apical membrane.

1.2.4 The Ontogenetic Developments of the Amino Acid Transport

Developmental studies of amino acid transport in the small intestine of various species has demonstrated that the timing and class of amino acid transporter appearance differs not only among the animal species but also at the various development stages of the same specie (Buddington & Diamond, 1989, 1990). Both herbivores and omnivores prefer high protein diet in their youth, a period when the absorption of essential amino acids is high. Because of some amino acids are in higher demand in adults making these conditionally essential amino acids. This is the case for arginine in puppies. On the oral-anal axis, one dramatic change is in colon, whereby adults

colon only transports electrolytes and water, but neonatal and fetal colon possesses many amino acid transport systems. On the crypt-villous axis, the youth cope with the higher amino acid transport load by creating a large surface area that increase nutrient uptake non-specifically. The enterocyte turnover rate of the villus tip is slower, the crypt cell migration is greater, and the crypt cells possess transport ability, all of which contributes to the increased mass of intestine possessing transport activity. Two mechanisms were proposed (Buddington & Diamond, 1989, 1990) to explain the control of amino acid transporter expression during animal development: (1) an external control mechanism by dietary changes in substrate or by some growth factors in diet (e.g. epidermal growth factor stimulating transport by enterocyte, paracrine/autocrines, secreted by salivary gland or Bruners glands or from food source such as milk); (2) an internal genetically hard-wired control mechanism that controls change independent of external environment.

1.2.5 Regulation of Intestinal Amino Acid Transport

The intestinal membrane amino acid transport systems are regulated by various factors, such as the animal development regulation discussed above, certain physiological states like pregnancy, or certain pathological states like disease diabetes, hyperthyroidism. Much attention was given to the regulation of transport activity at certain stage of

development by systemically circulating factors like hormones, or by the luminal composition like transporter substrates. The intestinal apical membrane amino acid transport regulation by transport substrate has been studied in vivo as described below.

Unlike other internal organs, in vivo studies show that the activities of the intestinal amino acid transporters are up-regulated by the dietary substrates they transport (Stevens, 1992a,b; Salloum et al 1990; Sharrer et al, 1981; Stein et al, 1987; Ferraris et al, 1988a,b; Diamond & Karasov, 1987; Ferraris & Diamond, 1989; Diamond, 1991). The substrates' unique pattern of up-regulation, their amplitude, and selectivity of each system's activity indicated that individual amino acid transporters are regulated independently by dietary substrates. Non-essential caloric amino acids up-regulate their transporter activities with increasing substrate. The essential, but potentially toxic amino acids regulate their transporter systems' activities in a different pattern. That is, at lower substrate concentration, the transporter activity decreases as substrate concentration increase; at higher concentration the transport activity increases as substrate concentration increases. A similar pattern is observed for sugar and dietary carbohydrate. The transport of non-essential amino acids is increased more by the dietary protein than that of the more toxic essential amino acids. This supports the notion that the absorption of

glucose, caloric and catabolic amino acids, and essential amino acids (possibly toxic if in excess amount) is regulated independently in vivo, which provides needed nutrients for the entire organism and which prevents substrate toxicity (Diamond, 1991). The mechanism of this induction has not been addressed hereafter.

Amino acids differ in their potencies to induce the same transporter. Although substrates generally make good inducers of their own transporters, there are some discrepancies (Levine, 1991; Diamond, 1991) between the inducers and transported substrates: transport unrelated amino acid is the best inducer.

The change in substrate-related transport activity is a relatively slow process. An increase in the luminal substrate level induces an reversible transport uptake capacity increase over the existing absorbing capacity by 2- to 10- fold within 24 hours. The substrate-specific up-regulation of nutrient absorption is directly related to the level of these substrate in the intestinal lumen. Lowering substrate levels causes the intestinal absorption capacity to decrease back down to the baseline level that appears to be genetically hard-wired (reviewed by Diamond, 1991). The down-regulation is a slow process (eg. 3 days for proline transporter in mouse).

Two mechanisms were proposed to explain the substrate-related intestinal amino acid uptake activity. The first is mucosal hyperplasia resulting in nonspecific uptake increase

(Laganiere et al, 1986; Diamond, 1991). Nontoxic nutrient exposure can induce a non-specific hyperplasia of the epithelium (increase epithelial cell numbers and size) and lengthen the villi to provide more absorbing capacity for all nutrients (Laganiere et al, 1986). The second explanation is that individual transporter activities are selectively increased as a result of the modification of transporter or/and increase the copies of transporters (James et al., 1987; Stein et al., 1987; Diamond et al., 1987; Scharrer et al., 1981) by the exposure of specific transport substrate.

In addition to the substrate regulation of transport activity, often conditions such as corticosteroid treatment and the conditions related to diabetes, hyperthyroidism, neoplasia, and pregnancy and lactation can induce intestinal mucosal hyperplasia (James et al., 1987; Levine, 1991).

In addition to absorbing nutrients for whole body needs, the small intestine enterocytes also require amino acids for their own proliferation, growth and differentiation. Epithelial cells rapidly turnover as enterocytes continuously migrate up from the immature proliferating crypt cells to become mature villous enterocytes along the crypt - villous axis. The supply of amino acids by membrane transport may be the rate-limiting step in the rapidly proliferating and protein synthesizing in undifferentiated cells (Seitz et al, 1989). Epidermal growth factor (EGF) and transforming growth factor-alpha (TGFα) also stimulate epithelia proliferation and

growth (Carpenter & Wahl, 1990). The relationship between the cell proliferation and the membrane amino acid transport is not clear.

Substrates not only regulate their absorptive activity, but are also vital to enterocyte health. Glutamine, transported via intestinal System B (Souba et al., 1992), is essential in preserving the intestinal mucosa (Souba, 1990). Glutamine deficiency cause impairment of intestinal mucosal barrier function (Souba et al, 1990). In this sense, glutamine regulates its transport activity through preserving a healthy state, in addition to its direct regulation of transport activity.

1.2.6 Molecular and Cellular Models of Amino Acid Transport Regulation

Several amino acid transport regulation models have been proposed for nonintestinal cell types. However, the knowledge of intestinal membrane amino acid transport regulation is still lacking.

One model for substrate adaptive regulation of System A in hepatocytes was proposed by Kilberg (1986). The model is based on the assumption that the rate of repressing System A independent of substrate concentration. The System A transporter protein synthesis process is controlled at the transcriptional level as a consequence of the equilibrium between positive and negative regulating factors: in the absence of extracellular amino acid and/or in the presence of

hormone, System A associated protein synthesis is stimulated (depressed), while in the presence of elevated intracellular amino acid levels, System A-associated protein synthesis is repressed by a regulatory protein.

Another model for adaptive regulation of System A in CHO-K_1 cells (chinese hamster ovary cell) was proposed by Englesberg group (1986). This model suggested that System A is regulated by at least two regulatory genes, R1 and R2. R1 produces an apopressor/inactivator (apo-ri) that is in equilibrium with a repressor/inactivator (ri). The elevated transported amino acids shift the apo-ri to ri1 which inhibits the transcription of the gene encoding System A transporter, and converts the existing transporter to an inactive state. R2 produces a constitutive repressor r2 which also negatively regulates the gene A. Insulin binds to its receptor and through an unknown pathway converts r2 to its inactive form. The absence of transported substrate and the presence of insulin have a synergistic effect on stimulating System A activity.

One study in a kidney cell line (MDCK) indicates the involvement of protein kinase C in System A regulation (Dawson & Cook, 1987).

Even though System A adaptive regulation was intensively studied, the molecular and cellular mechanisms of intestinal transport regulation of any amino acid are still unknown. The recent cloning of the System A cDNA (Kong et al., 1993) will

encourage regulation studies.

1.2.7 The Effects of Peptide Growth Factors in the Small Intestine

As members of the peptide growth factors family, Epidermal Growth Factor (EGF) and/or Transforming Growth Factor ∝ (TGF∝) each stimulate cell proliferation, protein synthesis, and cell differentiation in many cell types including the intestinal epithelial cells (Morrisset & Solomon, 1991; Carpenter & Wahl, 1990).

EGF is a 53 amino acid polypeptide, while TGF∝ is a 48 amino acid peptide. TGF∝ is structurally and biological functionally similar to the that of EGF. EGF is normally present in the intestine lumen from endogenous secretions from the salivary glands, the small intestinal Brunner's glands, autocrine/paracrine sources from the mucosa, or from exogenous sources such as milk and colostrum (Gaull et al.,1985; Britton, 1988; Potter, 1989). The sites for the EGF secretion to blood stream is unknown.

EGF and/or TGF∝ binds to the same EGF receptor in the plasma membrane, which is a member of the tyrosine kinase receptor family. The activated growth factor-receptor complex immediately phosphorylates the receptor itself and phosphorylates other substrates such as erb B2, ras oncogen, polyoma middle T antigen, or phospholipase C (PLC). The activated PLC alters inositol phospholipid metabolism leading to a elevated level of diacylglycerol (DAG) (Berridge, 1985;

Edelman et al, 1987; Klip & Douen, 1989), which activates intracellular protein kinase C. PK-C activates a series of unresolved mechanisms that ultimately result in cell division, proliferation and differentiation (Saier et al., 1988).

EGF receptors appear at both the luminal and basolateral membranes at a density gradient greater in immature crypt cells and less dense in villous enterocytes along the crypt -> villous axis (Hidalgo et al., 1989). This parallels the high proliferation rate in undifferentiated crypt cells (Pamukcum & Owens, 1991). Two-thirds of the EGF receptors are reportedly in the basolateral membrane (Reviewed by Brand, 1990). EGF/TGFα stimulate small intestinal epithelial proliferation, protein synthesis, and crypt cell maturation and migration toward villous tip cells. Recently, EGF receptor mRNA was identified in developing intestinal epithelial cells (Koyama & Podolsky, 1989). The EGF receptors reportedly existed at the apical and basolateral membranes of the human intestinal epithelial Caco-2 cell line (Hidalgo et al., 1989), with higher density in the undifferentiated cells compared to the differentiated cells. Two-thirds of the receptors expressed at the basolateral membrane. The K_m of the EGF receptors is 0.67 nM in Caco-2 cells (Hidalgo et al., 1989). Experiment data in our laboratory indicate that functionally EGF or TGFα each stimulates Caco-2 cell alanine and arginine transporter activities with similar potency when applied to either brush border or basolateral surfaces.

Even though the structure and biological functions of EGF and TGF∝ have been widely studied, the EGF/TGF∝ effects on intestinal amino acid transport has not been addressed.

1.3 The Human Intestinal Epithelial Cell Line (Caco-2 Cell Line)

The established intestinal epithelial cell line Caco-2 is derived from human colon adenocarcinoma cells (Fogh et al., 1977). It was originally used for in vitro colonic tumor studies.

Caco-2 cells can been grown on both solid plastic and porous filters for many sub-cultural generations. When grown on a solid surface, the Caco-2 cells form a confluent monolayer with tight junction and dome formation. Under normal cell culture conditions the confluent cells undergo a spontaneous enterocytic differentiation process (Pinto et al., 1983; Rousset et al., 1985). The biochemical and historical characteristics of the undifferentiated cells resemble those of the immature enterocytes, while the differentiated cells resemble the mature small intestinal epithelial cells. The differentiated Caco-2 cells become polarized, forming brush border apical membranes complete with peptide and carbohydrate hydrolases normally found as small intestinal apical marker enzymes. The enzymes include sucrase-isomaltase, lactase, trehalase, aminopeptidedase N, dipeptidylpeptidase IV, γ-glutamyltranspeptidase, and alkaline phosphatase (Pinto et al., 1983; Hauri et al., 1985; Rousset et al., 1985). The

undifferentiated sub-confluent cells are morphologically and biochemically equivalent to immature crypt cells, and differentiated post-confluent cells are undistinguished both morphologically and enzymatically from mature villus tip enterocytes (Hidalgo, 1988, 1989, 1990). The Caco-2 undifferentiated sub-confluent -> differentiated post-confluent state developmental steps mimic the enterocytes crypt -> villous maturation process. Many studies favorably recognize the Caco-2 cell line as an ideal in vitro analog of normal small intestine enterocytes (Zweibaum et al., 1983, 1991).

Organic solute transport studies on Caco-2 cells have revealed the same characteristics as those from other in vitro and in vivo small intestinal preparations (Blairs et al, 1987; Mohrmann et al, 1986; Nicklin, 1992). A few studies have been conducted regarding glutamine and proline transport characteristics in Caco-2 (Nicklin et al., 1992; Souba et al., 1992). These studies paralleled to those in other intestinal preparations.

In addition to characteristics indistinguishable from enterocytes, Caco-2 cells excel in providing a well-controlled homogenous population over a prolonged life span during cell development and differentiation. Uncontrolled adverse systemic factors found in vivo preparation are eliminated in the cell culture systems, so that the effect of a single variable can be studied in an unbiased setting. The Caco-2 cell line makes

it possible to study the nutrient transport and associated regulation over the enterocytes' entire developmental period. Nonetheless, Caco-2 cells are not entirely normal small intestinal epithelial cells. However, until the normal small intestinal epithelial cell model is established, the Caco-2 cell line provides the best in vitro human intestinal enterocyte model.

1.4 The Objective, Hypothesis, and Aims of the Present Study

1.4.1 The Objective

The overall objective of this in vitro study is to investigate the cellular basis of amino acid transport regulation in undifferentiated and differentiated states of a human intestinal epithelial cell line (Caco-2 cell line). This project concerns independent transporters serving structurally distinct amino acid substrates in the apical membrane of Caco-2 cells.

1.4.2 The Hypothesis

The hypothesis is that alanine and arginine are independently transported by discrete transporter systems in the Caco-2 apical membrane, and that the transporter activities are independently regulated in mature enterocytes and during enterocyte development. Dipolar L-alanine is transported via Na^+-dependent secondary active transport System B, while cationic L-arginine is transported via Na^+-

independent Systems y^+ in Caco-2 cells grown on solid surface or on porous membrane filters. We further hypothesize that the membrane's constitutive activities for System B and System y^+ each decrease over time during Caco-2 enterocyte differentiation and development. The activity of each transport system can be up-regulated above the constitutive level by two categories of regulating agents: (i) substrate analogues served by each transporter, and (ii) the peptide growth factors epidermal growth factors (EGF) and transforming growth factor (TGFα). Finally, we hypothesize that up-regulation of transporter activities occurs in two phases: an acute phased characterized by protein-synthesis-independent substrate trans-stimulation, and a chronic prolonged phase that likely involves protein kinase C and de novo protein synthesis.

1.4.3 The Specific Aims

Aim 1: To kinetically classify the alanine and arginine transport systems in the Caco-2 apical membrane and to examine the changes in the constitutive baseline transporter capacities of the sodium-dependent alanine transporter (System B) and the sodium-independent arginine transporter (System y^+) during the Caco-2 epithelial development and differentiation.

Aim 2: To examine the acute and the prolonged phases of individual amino acid substrates in increasing System B and System y^+ transporter capacities, in undifferentiated and

differentiated states.

Aim 3: To examine the roles of peptide growth factors TGF∝ and EGF and protein synthesis in changing System B and System y^+ activities in undifferentiated and differentiated states.

Aim 4: To examine the role of cellular protein kinase C in regulation of System B and System y^+ by substrate or TGF∝/EGF.

CHAPTER 2
GENERAL METHODOLOGY

2.1 Caco-2 Cell Culturing

The human intestinal epithelial Caco-2 cell is derived from human colon adenocarcinoma cells. The cells can be grown as a monolayer on both porous filters and plastic. Under normal cell culture conditions, Caco-2 cells can be subcultured for many generations. Some labs reports 90 or more passages. Caco-2 cell growth on the plastic surface is dependent on cell density. Cells divide horizontally, and cell attachment does not stop the cell growth as normally seen in cell culture. The attached cells continuously divide at a lower rates. Days later (depends on cell density, with higher cell density having a shorter turnover), the attached cells become confluent. The confluent state is represented by the cell to cell tight junction and by dome formation. The dome is caused by the unidirectional transport and trapping of water and electrolyte cross cell monolayer. Unique in the Caco-2 cells, the confluent Caco-2 cell undergo a spontaneous enterocytic differentiation process without changing cell culture conditions. The differentiating cells start to polarize by forming apical and basolateral membranes, with expression of the normal small intestinal epithelial cell

apical membrane marker enzymes on the cell membrane. To date, the biochemical and histological tests indicate that differentiated Caco-2 cells are quite similar to, but exactly like normal small intestinal epithelial cells. The timing and cellular characteristics associated with the differentiation process of the Caco-2 cells resemble those of the normal crypt to villous cell development. Caco-2 cells are a human colon tumor transformed cell line having 106 chromosomes. The unmistakable similarity of the histological and biochemical characteristics makes the Caco-2 cell line a ideal model for the in vitro analogue of the normal adult intestinal epithelium.

The confluency and differentiation of Caco-2 cell states are cell-attachment dependent. After trypsinization, the attached polarized cells detached to become single non-polarized cells, and lose their differentiated characteristics. Whether this process is a de-differentiation, or simply a turning off of existing differentiation expression, is still debatable. The daughter cells of these de-differentiated or undifferentiated cells then undergo another un-differentiation-confluency-differentiation process. Regardless the states of parent cells, newly divided daughter cells are undifferentiated. To ensure that the majority of cells are at the undifferentiated state, we have used cells only in their relative early generations (#19-50). The limited time of subculturing also reduce the possibility of mutation.

Cell culture studied have their advantages and limitations. On the positive side, cell culture provide a uniform environment. It is a relatively simple and straight forward preparation without the adverse effect of in vivo preparation. The experimental conditions are controllable. On the negative side, the cell line is not entirely normal cells. Furthermore cell culture conditions are not those of the in vivo physiological conditions, and there is the possibility of mutation. The cell conditions after subculturing may be different from the in vivo state. Despite the limitation of the cell culture, the Caco-2 cells are still considered to be an excellent model for adult intestinal epithelial study (Pinto et al., 1983; Hidalgo et al., Zwebaum et al., 1991)

For my study, transport studies in Caco-2 cells were performed in both the undifferentiated (age day 2-3) and the differentiated (age day 8-9) cells of the same subcultured batch of cells. In some cases in other cell ages (mentioned in text). Cell culture techniques are based on established procedures (Hidalgo et al., 1989; Blairs et al., 1987; Mohrmann et al., 1986; Pinto et al., 1983) and our modifications. The Caco-2 cells used for the present experiments were between the cell sub-cultured passages #19 - #50.

2.1.1 Materials

The established human intestinal epithelial cell line

Caco-2 was obtained from American Type Culture Collection, Rockville, MD. Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum, sodium bicarbonate, penicillin, streptomycin, non-essential amino acids, Trypsin/EDTA, and Dimethyl sulfoxide(DMSO) were from Sigma Co., St. Louis, MO. The 6-well Falcon tissue culture dishes and 100 mm tissue culture dishes were obtained from the Fisher Scientific, Pittsburgh, PA. The 0.2 μM medium filters were from Millipore Co. Bedford, MA. The 0.4 μm 24 mm Costar's Transwell-COL collagen treated microporous membrane filters (Catalog # 3425) were from Costar Co. Cambridge, MA. [^{3}H]-Alanine, [^{3}H]-arginine, [^{3}H]-glutamine and [^{3}H]-Threonine were obtained from Amersham Co., Arlinton Heights, IL. [^{3}H]-α-methyl aminoisobutyric acid was from American radiolabled chemicals Inc., St. Louis, MO. NaCl, choline Cl, KCl, $MgSO_4$, KH_2PO_4, $CaCl_2$, NaOH, and N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) (HEPES)/tris(hydroxymethyl)aminomethane (Tris) were obtained from Sigma Co., St.Louis, MO. Liquiscint scintillation fluid was from National Diagnostics, Atlanta, GA. The protein assay reagent was obtained from Bio-Rad Lab, Richmond, CA. Glacial acetic acid was from Fisher Scientific, Pittsburgh, PA. The scintillation counter and spectrophotometer were from Beckman, Irvine, CA.

2.1.2 Caco-2 Cell Culture

The Caco-2 cells were routinely grown on the 100 mm

falcon tissue culture dishes in 15 ml Sigma's Dulbecco's Modified Eagle Medium (DMEM; Sigma Co, St. louis, MO) containing 4.5 g/l glucose and 0.584 g/l glutamine, and supplemented with: 10% fetal bovine serum (Sigma Co, heat untreated catalog # F 4884)), 3.7% sodium bicarbonate, 100 IU/ml penicillin, 100 μg/ml streptomycin (Sigma Co., St louis, MO) and 1% non-essential amino acids (Sigma Co. St. Louis, MO). The original seeding cell density was 3 x 10^5 cells/ml, cells were counted using a hemacytometer. Cells were grown in a humidified incubator at 37°C in 10% CO_2/90% O_2. The day of seeding was designated as day 0. The growth medium was changed and cells were inspected daily.

2.1.2.1 Caco-2 cell subculturing

For the sub-culturing cells, cells four days old on the 100 mm dish were taken out of the incubator. The growth medium was aspirated, and cells were washed once with 37°C isotonic calcium-free saline solution containing 0.05% trypsin and 0.02% ethylenediamine-tetraacetic acid (EDTA; Sigma Co. St.Louis, MO) and immersed in 10 ml the same solution for 5 minutes in the sterile hood. The cell/trypsin mixture was dispersed with a narrow tip glass pipette (Fisher Scientific, Pittsburgh, PA) and the trypsin reaction was stopped by adding DMEM with 10% FBS. Then the dispersed cells were sedimented in a sterile conical centrifuge tube (Corning, Corning, NY) at 1000 x g for 5 minutes, and the supernatant was removed. Next, growth medium was added to re-suspend the cells (using a

narrow tip glass pipette) until cells were separated. Possible cell clumps were allowed to settle for a few minutes at 1 x g, and only the top layer of medium containing single clumped cells was used for sub-culture as confirmed by microscope examination. Cells then were seeded in three ways: (1) seeded in the 100 mm dish at a cell density of 3×10^5 cells/ml for future sub-culture purpose, (2) seeded in the 35 mm Falcon tissue culture dishes (6-well clusters; Becton Dickinson, Lincoln park, NJ)) at a density of 1.93×10^5 cell/ml for subsequent transport experiments, and (3) seeded in 24 mm porous filter at a cell density of 1.93×10^5 cells/ml for subsequent transport experiments. All cell culture procedures were performed under sterile conditions in a hood. All solutions used in cell culturing were filter-sterilized (0.2 μM membrane filter; Nalge, Rochester, NY; Millipak 20, Millipore Co, Bedford, MA), or/and autoclave sterilized. The growth medium, including the medium in the both upper and lower chambers of porous filter (Costar Co, Cambridge, MA), was changed daily. The cultures are inspected daily (using a phase contrast microscope) to monitor cell growth (dome formation, absence of contamination, etc).

2.1.2.2 Freezing Caco-2 cells

Four-day-old Caco-2 cells grown in the 100 mm dishes were trypsinized and centrifuged as described in above subculturing section. Cells were then re-suspended in 4°C DMEM containing 10% FBS and 5% dimethyl sulfoxide (DMSO; Sigma Co, St.Louis,

MO). One milliliter of the cell/medium mixture (10 million cells/ml) was then transferred into a sterile glass ampule or a Nunc plastic tube (Inter Med, Denmark), which was later sealed in a sterile hood. The sealed ampules and the Nunc tubes were immersed into a 4°C methyl alcohol freezing tank (Fisher Scientific, Pittsburgh, PA). The freezing tank was then placed into a -70°C freezer for 72 hours before the ample/tube were transferred into liquid nitrogen storage.

2.1.2.3 Re-thawing frozen Caco-2 cells

Sealed ampules containing the frozen Caco-2 cells from the American Type Culture Collection or liquid nitrogen storage were immediately immersed into a 37°C water bath until the ampule content was completely thawed. And the sealed ampules were immersed into 70% (v/v) alcohol for a minute. The following procedures were then performed inside a sterile hood. The ampules containing cells were opened and cells were transferred and suspended in 37°C DMEM containing 10% FBS. The cells were then sedimented in a conical centrifuge tube at 1000 x g for 5 minutes, and seeded in the 100 mm cell culture dishes following the procedures described in the above subculturing section. The re-thawing cells were grown for at least two subculturing generations before being used in any experiment.

2.2 Caco-2 Cell Monolayer Transport

The Caco-2 cell form a monolayer on both the plastic

surface and porous filters. For the pre-confluent cells, the junctions among cells are loose, and cell membrane has not polarize yet. The cell uptake may involve the membrane, excluding the portion attached to the plastic surface. For the differentiated state, cells have already polarized with basal membranes attached to plastic surface or filter; lateral membranes are formed beneath the tight junctions which connect the apical membranes. The apical surface faces the outer environment. Organic solutes enter cells through the apical membrane, so that the para-cellular pathway is minimal. This has been confirmed by [^{3}H]-inulin extracellular studies (Arturson et al., 1992).

The membrane transport of amino acid is a bi-directional process. The measured transport activity is the net influx of transport equivalent to vectorial difference between the two unidirectional fluxes. The rate of the net is therefore determined by the total flux during a period of time during which the flux is linear. In the case of monolayer transport, the rate of the membrane transport of amino acid is therefore equal to the rate of net accumulation of amino acid within the cells over a period of time. We measured the total accumulation of amino acid and the time at which the amount accumulation is linear proportional to the accumulation time. The mode and characteristics of membrane transport of amino acid are determined by the Menten-Michaelis kinetic analysis.

The amino acid transport experiments were performed on

the 35 mm falcon dishes (6 well clusters) at the cell passages #17 - #50.

2.2.1 Caco-2 Cell Monolayer Transport

The amino acid uptake experiments were performed at room temperature (22.°5C ± 1.0°C). Cells were taken out of the incubator. Then the growth medium was aspirated, and cells were rinsed three times with uptake buffer (22.5°C) containing 137 mM NaCl (or choline Cl), 10 mM HEPES/Tris (pH 7.4), 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, and 2.5 mM $CaCl_2$. The uptake was initiated at time = 0 by adding 1 ml uptake buffer plus 0.01 -10 mM [^{3}H]-alanine (2 μCi/ml, isotope was dried in nitrogen gas first and was then re-hydrolyzed in the uptake buffer), 0.005 - 5 mM [^{3}H]-Arginine (2 μCi/ml), or other isotopes and concentrations as mentioned below into the cell monolayer. For the System B regulation experiments, 1 mM unlabeled MeAIB was also added to the uptake buffer to block possible alanine uptake via the System A. For the arginine uptakes, 10 mM unlabeled leucine was added to the uptake buffer to block the System $b^{0,+}$. During the uptake procedures, the cell cultures were continuously shaken by an orbital shaker (1 Hz). The uptakes were stopped by aspirating the uptake buffer, and then adding 2 ml ice-cold uptake buffer (lacking substrate) immediately to the cells. The ice-cold buffer is aspirated, cells were rinsed two more times using the same ice-cold buffer. Isotope was extracted from the cells

by adding 1 ml 1N NaCl to the cells. After overnight extraction (continuous shaking), a 200 µl aliquot of NaOH extract was added to 10 ml Liquiscint scintillation fluid which was then neutralized with 200 µl glacial acetic acid. Radioactivity (counts per minute, CPM) was measured in the Beckman scintillation counter with quench normalized using the "H-number" method. Because the nonspecifically adhering label was < 1 % of the total counts per minute (CPM) associated with the cell uptake, the double label ^{14}C-inulin space measurement was not performed for subsequent experiments. The protein content in the NaOH extract was measured by the Bio-Rad protein assay. The rate of amino acid uptake activity was equivalent to the initial linear slope of the uptake time course. All subsequent uptake experiments were conducted within the linear period at a uptake period < 10 minutes, with the 0 minute points serving as blanks. The amino acid uptake rates are expressed as nmole amino acid/mg cell protein/minute. The sodium-dependent alanine transport is equivalent to measuring total alanine uptake in NaCl buffer and in choline Cl buffer, and then subtracting the difference.

Bio-Rad Protein Assay

The Bio-Rad protein assay reagent was diluted 1 : 4 with de-ionized water. Fifty microliter cell/NaOH mixture was added into the diluted reagent (5 ml), with 50 µl 1.4 mg/ml standard γ-globulin as the protein reference (plus additional 50 µl 1

N NaOH) and the 5 ml diluted reagent plus 50 μl 1 N NaOH as the blank. The samples and reagents were mixed well and stabilized for 15 minutes. The protein absorbance was measured at wavelength of 595 nm by using the spectrophotometer. The sample protein contents were then calculated by:
sample protein = (sample absorbance x 1.4 mg/ml) ÷ (standard protein absorbance).

2.2.2 Radioactivity Measurement

Cell/NaOH aliquot (200 μl) was added into a 20 ml vial and was neutralized by adding 200 μl glacial acetic acid. Liquiscint scintillation cocktail (10 ml) was added to the mixture. A 200 μl sample of uptake buffer (containing a known specific activity of isotope), 200 μl 1N NaOH, and 200 ml glacial acetic acid were added together with 10 ml liquiscint in the 20 ml vials. The vials were then placed into the Beckman scintillation counter. The [^{3}H]-radioactivity was obtained as counts per minute. Uptake was subsequently calculated as nmole amino acid/mg protein/minute.

2.3 Monolayer Transport In Caco-2 Grown On Porous Filters

As mentioned above, the Caco-2 cells can be grown on either the plastic surface or porous filters. The confluent cell monolayer on porous filters provide additional dimensions to the membrane transport studies. The confluent cell monolayer forms a barrier separating the upper chamber and

lower chamber of the filters. The confluency is determined by measuring the electrical resistance across the cell layer. The apical membrane is accessible via upper chamber and the basolateral membrane is accessible to the lower chamber. Using cell monolayers grown on plastic we measured only the flux of amino acid across the apical membrane to cell cytosol. By using the porous filter, we can not only measure the flux from apical to cytosol, but also flux from basal to cytosol, and therefore the trans-cellular fluxes from apical to basal or basal to apical side. Thus we can integrate the events at basal or apical surfaces, including regulatory receptors, and site sites of transport. The membrane transport characteristics were determined by kinetic analysis in both basolateral and apical membranes.

2.3.1 Transport In Caco-2 Grown On Porous Filters

The uptake experiments were performed at room temperature (22.5°C ± 1.0°C). The Caco-2 cell culture was taken out of the incubator. The trans-epithelial resistance was measured using a trans-epithelial open circuit potential difference apparatus (world precision instrument Inc, New Haven, CT), confluent cells with a resistance ≥ 300 Ω • cm^2 were used for uptake studies. The growth medium in both the upper and lower chambers was aspirated, and the cells were rinsed three times with uptake buffer (room temperature) containing 137 mM NaCl (or choline Cl), 10 mM HEPES/Tris (PH 7.4), 4.7 mM KCl, 1.2 mM

$MgSO_4$, 1.2 mM KH_2PO_4, and 2.5 mM $CaCl_2$. These experiments were conducted in collaboration with Dr. S. Smith of Burroughs-Wellcome company (Research triangle, NC).

For the measurements of the apical-cellular-basolateral amino acid movement, the amino acid uptake was initiated at the time = 0 by adding 1 ml uptake buffer plus [^{3}H]-alanine or [^{3}H]-arginine into the apical side (the upper chamber), with the basolateral side exposed to 3 ml uptake buffer (lacking amino acids) in the lower chamber. During the uptake period, the cell cultures were continuously shaken by an orbital shaker (1 Hz). The uptakes were stopped by aspirating the uptake buffer, taking the filters out of lower chamber, and adding 2 ml ice-cold uptake buffer (lacking substrate) immediately to the upper chamber. The buffer was aspirated and cells were rinsed by the ice-cold buffer three times. Isotope trapped inside the cells was extracted from the cells by adding 2 ml 1 N NaOH to the cells (filters were first cut out off the dishes). After overnight extraction (continuous shaking), a 200 μl aliquot of NaOH extract was added to 10 ml Liquiscint scintillation fluid which was neutralized with 200 μl glacial acetic acid. Isotope trapped in the lower chamber was measured by transferring 200 μl of this buffer to 10 ml Liquiscint. The radioactivity was counted in the Beckman scintillation counter.

For the measurement of basolateral-cellular-apical movement, uptake was initiated at time = 0 by immersing

filters (with 1 ml uptake buffer with no substrate in the upper chamber) into 3 ml uptake buffer plus [^{3}H]-alanine or [^{3}H]-arginine in the lower chamber. During the uptake period, the cell cultures were continuously shaken by the orbital shaker (1 Hz). The uptake was stopped by removing the filters out of the lower chamber, and removing the upper chamber immediately. The filters were rinsed three time with ice-cold buffer. Isotope trapped in the cells and in the upper chamber buffer was measured separately as described above.

For the measurement of the apical- basal trans-cellular amino acid movement, the uptake was initiated at time = 0 by adding 1 ml uptake buffer plus [^{3}H]-alanine or [^{3}H]-arginine to the apical side and immersing the filter into 3 ml buffer (lacking substrate) in the lower chamber. Uptakes were stopped by removing filters from the lower chamber. The isotope accumulated in the lower chamber was the measured as described above.

For the measurement of the basal-apical trans-cellular movement, the uptake was initiated at time = 0 by placing the filter (with 1 ml uptake buffer with no substrate in the upper chamber) into 3 ml buffer containing [^{3}H]-alanine or [^{3}H]-arginine in the lower chamber. During the uptake period, the cell cultures were continuously shaken (1 Hz). The uptakes were stopped by taking out the buffer from the upper chamber. The isotope accumulated in the upper chamber was measured in scintillation counter as described above.

2.3 Statistical Analysis

All experiments were conducted in triplicate (including the 0 time blanks, and the uptakes in Na^+ and choline buffers) and all experiments were confirmed in at least two differently seeded generations of cells. Curve fitting was conducted by non-linear regression analysis. Values were reported as the mean $\pm$ SE. ANOVA used for statistical analysis with Duncan's test, the level of significant $p \leq 0.05$.

CHAPTER 3
CLASSIFICATION OF THE ALANINE TRANSPORT SYSTEMS IN THE CACO-2 CELL MEMBRANE

3.1 Introduction

The characterization of membrane amino acid transport was pioneered by Christensen several decades ago (Christensen et al., 1952). Since that time, the criteria to assess a membrane amino acid transport systems in mammalian membranes have indeed substrate specificity, ion-dependency, initial uptake rate kinetics, patterns of analogue cross-inhibition of amino acid uptake, and exclusive substrates transported through specific transport systems. The development concept of the Na^+-gradient-driven, secondary active solute transport (Crane et al., 1962) was an another important addition to describing the transport phenomena. In the past several years, there have been reports of cloning cDNA encoding several amino acid transporters (Kilberg et al., 1993), and cloning of possible regulatory proteins for Systems L, $b^{0,+}$ or y^+. There has no cloning, antibody production, nor purified transporter protein reported for major alanine transport systems in intestine. The phenomenological criteria remain the sole tools to classify alanine transport systems. The sole exception is the recent cloning of System A (Kong et al. 1993), although System A

represents a minor pathway in intestine (described below).

The major membrane transport Systems A, ASC, $B^{0,+}$, B, L, and asc for the dipolar amino acid L-alanine have been intensively studied in various tissues. (Oxender et al., 1963; Christensen et al., 1963; Stevens et al., 1982; Van Winkle et al., 1985, 1987, 1988; Van Winkle & Campione, 1990; Kilberg et al, 1993; Stevens, 1992a,b). Among these alanine transport systems, only System B was originally described as an unique in intestinal epithelial cell transporter (Stevens et al., 1982, 1984). The others systems were first described for non-epithelial cells (Stevens, 1992a,b).

System A is a strictly Na^+-dependent system which is broadly selective for most dipolar (neutral) amino acids (Christensen et al., 1963). System A is found in many tissue membranes. One specific feature of System A is that the non-metabolized MeAIB is a specific substrates for System A. AIB serves as less specific substrate. System A regulatory properties have been intensively investigated in hepatocytes and other tissue. Several regulation mechanisms of System A activity were proposed (Kilberg, 1986; Engleberg, 1986; Dawson & Cook, 1987). System A reportedly exists at the apical and basolateral membrane of guinea pig intestinal epithelia (Hayashi et al., 1980; Del Castillo et al., 1991).

System ASC is another Na^+-dependent transport system serving 3- and 4-carbon neutral amino acids, exemplified by alanine, serine and cysteine (Kilberg et al, 1981). System ASC

is distinct from System A in that System ASC does not transport MeAIB. System ASC is also found in many tissue. System ASC reportedly exists at guinea pig intestinal epithelial apical membrane (Hayashi et al., 1980).

System $B^{0,+}$, first described in mouse blastocytes (Van Winkle et al., 1985), is a Na^+-dependent transport system that transports both the cationic and neutral amino acids. System $B^{0,+}$ is expressed in many tissues.

The strictly Na^+-dependent transport System B exclusively found in the apical membrane of the epithelial cells. It was first described in rabbit jejunum apical membrane vesicles as a broadly selective system serving neutral amino acids (Stevens et al., 1982, 1984, Stevens, 1992a,b). It was originally named System Neutral Brush Border (NBB), and later was renamed as "System B" (Stevens, 1992a). System B substrate selectivity is similar to the System $B^{0,+}$ in that both are Na^+-dependent, as it possesses a broad selectivity for most dipolar amino acids. Both Systems B and $B^{0,+}$ interact with the bicyclo-amino acids 2-amino-2-norbornanecar-boxylic acid (BCH) or BCO. However, System B $^{0,+}$ is inhibited by cationic amino acids such as lysine and arginine, while System B is not interactive with cationic amino acids. System B may possible be a variant of System $B^{0,+}$.

The Na^+-independent System L was shown to exist in the apical and basolateral membrane of many cell types. System L is a broadly selective system serving neutral amino acids,

cysteine, phenylalanine, and BCH.

System $b^{0,+}$, a Na^+-independent analogue of System $B^{0,+}$ serving neutral and cationic amino acid such as lysine and arginine. The substrate scope of System $b^{0,+}$ is similar to System $B^{0,+}$. System $b^{0,+}$ exists in many cell types, but it has been previously reported in the intestinal membranes.

Only a few organic solute transport studies in the Caco-2 cells have been reported (Blais et al., 1987; Hidalgo et al., 1988; Mohrmann et al., 1986; Souba et al., 1992, Nicklin, 1992). Each of the reports showed the similarity of solute transport characteristics in Caco-2 cells and intestinal epithelial cell.

Inasmuchas alanine transport systems were not described for Caco-2 culture, our study began by describing the alanine transport systems in the Caco-2 monolayer.

3.2 Methods And Materials

3.2.1 Methods

The [^{3}H]-alanine uptake experiments were performed in both the pre-confluent (day 2 - 3) and confluent (day 8 -9) cells. The basic uptake procedures were as described above (chapter 2). Special uptake conditions are presented below where appropriate.

3.2.2 Materials

The materials were as the same as discussed in the chapter 2.

3.3 Results

3.3.1 Alanine Uptake Time Course

The 50 μM and 5 mM [^{3}H]-alanine uptakes were measured at during various times (0 - 45 minutes) in the uptake media containing 137 mM NaCl or 137 mM choline Cl. The 50 μM alanine uptake on day 2 cells was shown at Fig. 3-1. The accumulation in the NaCl medium was greater at any point than that in the in choline Cl medium suggesting a Na^+-activation phenomenon. The initial alanine accumulation in the Caco-2 monolayers was linear up to 15 minutes (at both [^{3}H]-alanine concentrations of 50 μM and 5 mM). Since the transport activity was expressed as the alanine uptake rate measured during the linear period, the uptake period of 0 - 10 minutes was chosen for all the subsequent uptake measurements. The rate was equal to total accumulation divided by the time period.

3.3.2 Alanine Uptake Rates at Various Caco-2 Cell Ages

The 50 μM [^{3}H]-alanine uptake rates were measured at various Caco-2 cell ages ranging from 1 day old to 35 days old in both the NaCl and choline Cl media (Fig. 3-2). At each cell age, the total alanine uptake rate in NaCl medium was

consistently greater than that in the choline Cl medium. This difference was greater in the younger cells, compared to confluent cells.

Alanine uptake rates in both the NaCl and choline Cl media decreased as cell age increased. The uptake rates in NaCl medium decreased rapidly over a period of several days (≤ 4 days old), and maintained steady after differentiation (≥ 9 day old). The decrease in alanine uptake in choline Cl medium was less dramatic than that in the NaCl medium, with the rate consistently decreasing throughout the cell ages.

In a separate study, the 24 hours proliferation rates of Caco-2 cells (2 day old - 14 days old) were measured by incubating the cells in [^{3}H]-thymidine medium for 24 hours. The amount of thymidine incorporated into the cells during the period represented the cells relative proliferation rate. The thymidine incorporation into the Caco-2 cells deceased as cell age advanced (Fig. 3-3).

The pattern alanine uptake rates at various cell ages was coincident with the cell proliferation rates.

3.3.3 Ion-dependency

The uptake of 50 μM [^{3}H]-alanine was measured in the uptake media containing 137 mM NaCl, 137 mM choline Cl, 137 mM KCl, or 137 mM LiCl. The total alanine uptake rate in the NaCl medium was 8-fold greater than that in either the choline Cl, KCl and LiCl media at [alanine] = 50 μM (Fig. 3-4). The total

alanine uptake rates in the choline Cl, KCl, or LiCl media were not significantly different. Alanine uptake in the NaCl uptake medium was not significantly different from that in the medium containing sodium gluconate.

These data indicated that alanine uptake activation was strictly Na^+-dependent. Other cations such as Li^+ or K^+ could not substitute Na^+ in activating alanine transport. Chloride was not required for the Na^+-activation.

For all the subsequent experiments, the Na^+-dependent fraction of alanine total uptake was obtained by subtracting the uptake measured in choline medium from the total uptake measured in sodium medium.

3.3.4 The Effect of pH on Alanine Uptake

The uptake of 50 μM alanine in both the NaCl and choline Cl uptake media was measured at various media pH ranging from pH 6.0 to pH 8.5. HEPES and Tris were used to adjust media pH. The total alanine uptake rates in both the NaCl and choline Cl media increased steadily as the medium pH increased (pH = 6.1, 7.4, and 8.5) (Fig. 3-5).

3.3.5 Na^+-Activation of Alanine Uptake in Caco-2 Cells

The uptake of 50 μM alanine was measured in the uptake media containing NaCl ranging from [NaCl] = 0 mM to 137 mM (choline Cl as substitute). The total alanine uptake rates at both day 2 and day 9 cells increases as media NaCl

concentration increase. The uptake rates as a function of NaCl concentrations gave a hyperbolic shape (Fig. 3-6). The non-linear regression analyses of the Hill equation gave the same Na^+-activation Hill coefficient (n = 1) at each cell age, while the V_{max} was greater in day 2 cells than in day cells. The same Hill coefficient (n = 1) indicates that one Na^+ binds to transporter coupled with each alanine molecule transported, in cells 2 days and 9 days old.

These data indicated that the alanine uptake capacity was greater in day 2 cells. Therefore, the difference in transport capacity between day 2 and day 9 cells was not due to the transport system's affinity for Na^+-activation coefficient changes.

3.3.6 Alanine Uptake Kinetics

The alanine uptake in uptake media containing 137 mM NaCl or containing choline Cl was measured at various [^{3}H]-alanine concentrations ranging from 10 μM to 5 mM at the cell ages of day 2 and day 9. The kinetics at day 2 and day 9 cells were shown as uptake rates measured as a function of alanine concentration (Fig. 3-7; Fig. 3-8). The kinetics in either the NaCl or choline Cl medium each displayed both saturable and non-saturable components, indicating multiple transport systems were involved. At each alanine concentration, the uptake rate was higher in the NaCl medium.

In the choline Cl media, alanine uptake occurred via two

pathways: a saturable Na^+-independent carrier-mediated system and non-saturable simple passive diffusion. In NaCl media, besides the two pathways discussed in the choline Cl medium, an additional saturable Na^+-dependent system exists.

For the non-saturable component, the passive permeability coefficient (P) describing the relation $J = P \cdot [Ala]$ was constant at $0.53 \pm .08$ μliter/(mg protein)/min regardless of the cell differentiation states.

For the saturable components, non-linear regression analyses of Na^+-dependent alanine transport kinetics gave K_m = 164 ± 26.1 μmole alanine and V_{max} = 2.79 ± 0.21 nmole/mg protein/min for the day 2 cells. For 9 day old differentiated cells, the K_m was 159.0 ± 13.6 μmole alanine and V_{max} was 0.512 ± 0.03 nmole/mg protein/min (Fig. 3-9). Regarding the Na^+-independent alanine uptake system (tentatively, System L), the activity decreased from V_{max} = 1.85 ± 0.25 nmole/mg/min in the undifferentiated (day 2) cells to V_{max} = 0.38 ± 0.017 nmole (mg protein)$^{-1}$ min^{-1} in the differentiated (day 9) cells. The System L apparent K_m for alanine was unaffected by cell age (differentiated cell K_m = 1.10 ± 0.19 mM vs. differentiated cells K_m = 1.02 ± 0.007 mM alanine).

The kinetics revealed that alanine uptake capacity was higher in day 2 cells than that in day 9 cells, and therefore the difference was only a V_{max} effect. The affinities of both the saturable components were not affected by cell ages. The non-saturable passive diffusion was not affected by cell age.

3.3.7 The Analogue Cross-inhibition Pattern

The 50 μM [^{3}H]-alanine uptake rates were measured in media containing 137 mM NaCl and 137 mM choline Cl plus 5 mM single amino acid analogues (natural amino acids, BCH, MeAIB, AIB, and β-alanine with 5 mM mannitol as control).

For the Na^+-independent alanine transport system, alanine uptake was strongly inhibited by phenylalanine, alanine, leucine, threonine, serine, glutamine, asparagine, cysteine, and BCH, and weakly by MeAIB, AIB, and glycine. Lysine, and glucose did not inhibit (Fig. 3-10 & Fig. 3-11).

For the Na^+-dependent alanine transport system, the uptake activity was inhibited by 5 mM amino acid analogues (natural AAs plus BCH, MeAIB, and β-alanine) was shown in Fig. 3-12. The Na^+-dependent [^{3}H] alanine transport was strongly inhibited by threonine, glutamine, serine, cysteine, and asparagine. Weaker inhibition was elicited by glycine, phenylalanine, leucine and the bicyclo amino acid BCH. MeAIB and cationic amino acids elicited <10% inhibition. Dixon inhibition analyses indicated that the glutamine inhibition was classic competitive inhibition, while the MeAIB affect was un-competitive (Figs. 3-13 - 3-15). Proline, glycine, and phenylalanine gave high K_i values (Figs. 3-16 - 3-21).

The pattern and degree of amino acid analogue inhibition of the Na^+-dependent alanine uptake was identical at both cell ages, suggesting that the same transporter system was operative regardless of the cell age (Fig. 3-22).

In a separate study, the 50 μM [^{3}H]-MeAIB uptake rates were < 5% of the same concentration of alanine, suggesting a minimal contribution by System A in our Caco-2 cells line.

3.3.8 Alanine Uptake on Porous Filters

Uptake of alanine (50 μM) into the apical and basolateral surfaces of confluent Caco-2 monolayer grown on porous filters were measured. The Caco-2 cell monolayer confluency was determined by measuring the trans-cellular resistance, with the trans-epithelial resistance $\geq$ 300 Ω x cm^2 was considered confluent. The apical compartment to the basal compartment of 50 μM alanine uptake was measured (Fig. 3-23). The majority of alanine across the apical membrane was accumulated inside the cells rather than transport across to the basal side (Fig. 3-24).

Alanine (50 μM) uptake at the basal membrane to cytosol and apical compartment was also measured (Fig.3-25, 3-26, and 3-27). The uptake in NaCl medium was greater than that in the choline Cl medium, indicating a Na^+-activation event.

3.4 Discussion

The alanine uptake in Caco-2 monolayer at the different cell ages was studied. The alanine uptake activity was different at various cell age, indicating the possible regulation of cell development. There were several pathways for alanine uptake. By using the membrane transport system

classification criteria, we classified the alanine transport systems in Caco-2 cells, as discussed below.

3.4.1 Alanine Uptake Activity vs Cell Ages

Both the Na^+-dependent and Na^+-independent alanine uptake activities decreased as cell age increased at the alanine concentration of 50 μM (over the cell age span of 1 - 35 days) (Fig. 3-2). What were the mechanisms underlie this cell development regulation? There were several possible mechanisms that could underlie this development-related regulation. Non-specific membrane potential or other membrane property change could cause a non-specific driving force alteration, permeability of the membrane could change, or specific functional change of specific transport systems could occur. Each of these possibilities was explored.

The Na/glucose cotransport activity on Caco-2 monolayer has been reported to increased with cell age (Blais et al., 1987). The opposite direction of alanine uptake activity and Na/glucose activity with cell age rule (Fig. 3-23) out the possibility that the age-associated transport effect was due to the non-specific membrane electrochemical gradient which may associated with cell age. Therefore the non-specific driving force was not likely to be involved in the mechanism.

In terms of membrane properties at different cell ages, our kinetics studies gave the same diffusion permeability coefficients in day 2 and day 9 cells, even though the alanine

uptake rates were several fold higher in the day 2 cells (Fig. 3-2). The alanine uptake change over the cell ages was therefore only a portion of saturable carrier-mediated uptake. The membrane permeability was then unlikely to be involved in the regulation mechanism.

As discussed above, the alanine uptake rates decreased with advancing cell age, while Na/glucose cotransport increased with cell age (Fig. 3-23). This opposite direction of transport activity suggests that the function of alanine and glucose were not the same in cell development. Alanine was not solely for caloric purpose. In the light of the cell proliferation rate decrease with the cell age increase (Fig. 3-3), the reduced alanine uptake may be due to the lowered requirement for amino acids, but not for glucose. These data also indicated that the Na/glucose and alanine uptake in Caco-2 cells were independently regulated by the cell differentiation and development.

3.4.2 Classification of the Alanine Transport Systems

There were three alanine transport pathways in Caco-2 monolayers for alanine at both cell stages (the undifferentiated and the differentiated stages): a simple passive diffusion, a Na^+-independent system, and a Na^+-dependent system.

3.4.2.1 Simple Passive diffusion

The same passive permeability coefficient measured in

both the day 2 and day 9 cells suggested that the Caco-2 cell development did not alter the membrane permeability to alanine. The diffusion rates of alanine across the cell membrane at certain alanine concentrations were the same at either cell ages. The passive diffusion contribution at 50 μM alanine uptake was minimal, less than 1% of total uptake in NaCl. At higher alanine concentrations, the passive diffusion contribution was greater. At [alanine] = 5 mM, passive diffusion contributes 90% of total alanine uptake in NaCl.

3.4.2.2 Na^+-independent transport system is System L

One saturable Na^+-independent alanine transport system existed in both the day 2 and day 9 cells. The observed activity decreased with the advancing cell age (Fig. 3-2). The activity decrease was coincident with the cell proliferation rates (Fig. 3-3). The activity was possibly regulated by cell proliferation requirements.

The Na^+-independent alanine transport kinetics in both the 2 day old and 9 day old cells revealed that the transport activity V_{max} was higher in the day 2 cells. The transport apparent affinity K_m was the same at both cell ages. These kinetic parameters strongly indicate that the transport capacity was greater in the day 2 cells; the activity change was a V_{max} effect, not K_m effect, suggesting the presence of same transport system in both differentiated and undifferentiated states. The activity change was likely a change of copies of functional transport units in the membrane

instead of some modification of transporter affinities.

The amino acid analogue inhibition patterns on both the day 2 and day 9 cells were similar in that phenylalanine, leucine, BCH, and alanine strongly inhibited the alanine uptake, while MeAIB, glycine and lysine were weak inhibitors (Figs. 3-10, 3-11). These inhibition patterns strongly resemble that of the System L. The non-interaction with lysine ruled out the possibility of the System $b^{0,+}$. The strong BCH inhibition suggesting the unlikely System asc.

Based on the kinetic characteristics, the pH sensitivity, and the cross-inhibition pattern, We conclude that the Na^+-independent alanine uptake is via the System L at both the day 2 and day 9 cells.

3.4.2.3 Na^+-dependent alanine transport System B

The alanine uptake in the Caco-2 cells was strongly Na^+-dependent in either day 2 old and day 9 old cells (Fig. 3-4). The Na^+-dependent portion was more than 85% of the total alanine uptake in NaCl medium at 50 μM alanine (Fig. 3-4). No other monovalent cationic K^+ or Li^+ substitute for the Na^+ in activating the alanine uptake. Furthermore, the system was not activated concomitantly by Cl^-.

One of the important aspect in classifying transport systems was the cross-inhibition profile. The amino acid analogue inhibition pattern of the Na/alanine uptake for both the day 2 and day 9 cells was similar: the Na/alanine transport was strongly inhibited by threonine, serine,

glutamine, cysteine, and asparagine; Weaker inhibition was elicited by glycine, phenylalanine, leucine and BCH. MeAIB and cationic amino acids elicited < 10% inhibition (Fig. 3-12 & 3-22). We can compare this inhibition pattern with the amino acid inhibition patterns of the known Na/alanine Systems A, ASC, B, and $B^{0,+}$. System A is a strictly Na^+-dependent system selective for dipolar amino acids including alanine. Many neutral amino acids competitively inhibit Na/alanine transport via System A. One special aspect of System A is its unique ability to transport MeAIB. In our inhibition study, MeAIB blocked less than 10% of the Na/alanine transport activity (Fig. 3-12). Dixon analysis revealed that the MeAIB inhibition was a non-competitive inhibition (Fig.3-15). These combined data exclude System A as a major transport system in Caco-2 cells.

System ASC, the Na^+-dependent system serves short-chain neutral amino acids alanine, serine, and cysteine. In our study, serine, cysteine strongly inhibited alanine/Na uptake. However, phenylalanine and glycine, two competitive inhibitors of System ASC did not inhibit the Na/alanine transport in our study, as it would for the classic System ASC (Figs. 3-12, 3-18-21). Based on this and the similarity of our data to System B (discussed below), we exclude System ASC as the transport system. Because the characteristics were very close, however, definite classification is not possible without more precise test methods such as cDNA probes or antibodies.

Another Na^+-dependent alanine transport system is System $B^{0,+}$, which serves both the neutral amino acids and cationic amino acids. The only evidence that does not support existence of System $B^{0,+}$ was that cationic amino acids arginine and lysine did not inhibit Na/alanine uptake in our study (Figs. 3-12 & 3-22). Thus, it is unlikely that System $B^{0,+}$ exists in Caco-2 cells.

The final Na/alanine system candidate System B described first for intestinal epithelial cells (Stevens et al. 1984). System B has only been found in the epithelial cells of vertebrate and invertebrates (Stevens, 1992). The substrate selectivity of System B is very similar to System $B^{0,+}$ except that System B does not interact with cationic amino acids (Figs 3-12 & 3-22). Na/alanine uptake was strongly inhibited by of neutral amino acids serine, threonine, cysteine, weakly by glycine and phenylalanine, interaction with BCH, and was interactive with cationic amino acids arginine and lysine in our studies (Figs. 3-12 & 3-22). The amino acid analogue inhibition pattern supports the existence of System B. The apparent affinity K_m = 159 μmole alanine in Caco-2 was similar to the System B report elsewhere (Stevens et al., 1982) (Fig. 3-9). Furthermore, the pH sensitivity (Fig. 3-5) and the Na^+-activation Hill coefficient (n = 1) (Fig. 3-6) further support the case for System B. Based on the our evidence (Figs. 3-4 through 3-9, and 3-11 through 3-22), we conclude that the Na/alanine transport system in the Caco-2 cells was likely

System B.

The System B transport activity decreases as cell age increases (Fig. 3-2). The decrease in activity was coincident with the decrease of cell proliferation rates with cell ages (Fig. 3-3). The proliferation rate may be related to the cell requirement for amino acid. In contrast to the Na/alanine transport activity, the Na/glucose activity increases as cell age advances (Blais et al., 1987) (Fig. 3-23). These opposing changes in activity for Na^+-dependent solute transport as a function of cell ages excludes the likelihood that the amino acid transport was regulated by non-specific membrane electrochemical potential effects. Furthermore these data suggest that cell development is associated with the independent regulation of amino acid and glucose transport systems. We tested the Na/alanine transport characteristics in two different cell states, the undifferentiated state (day 2 cells) and differentiated state (day 9 cells). The amino acid analogue inhibition pattern, pH sensitivity, and Na^+-activation Hill number were the same for both the day 2 and day 9 cells (Figs. 3-4, 3-5, and 3-12). The transport kinetics gave a V_{max} on the day 2 that was greater than for day 9 cells, while the apparent affinity K_t was the same on both cell ages (Fig. 3-9). All the transport characteristics of the Na^+-dependent alanine transport (except V_{max}) were identical in both the undifferentiated and differentiated states, suggesting that the same transporter system was operative

regardless of the cell age. The kinetics also indicated that the transport capacity was greater in the day 2 cells, and that the activity difference between the two days was like caused by the change in functional transporter units expressed in the membrane, rather than modification of existing transporter affinity.

3.5 Summary

Alanine is transported in Caco-2 cell by a Na^+-dependent transport System B, a Na^+-independent transport System L, and simple passive diffusion. These same systems were operative in both the undifferentiated and differentiated cell states. The passive diffusion coefficient was not affected by cell development. The alanine transport Systems B and L activities are down-regulated as the cell develops, coincident with the cell proliferation rates. The decrease in transport activities are likely caused by the decrease in copies of functional transporter units, rather than modification of existing transporter affinity for substrate or ions.

Fig. 3-1. Alanine uptake time course

The uptake of alanine (50 μM and 5 mM) was measured in uptake media containing NaCl and choline Cl in day 2 and day 9 cells. The alanine uptake in NaCl media was greater than that in the choline Cl media at any point (except $t = 0$, $p < 0.05$, $n = 6$). The uptake values in this figure and subsequent experiments were expressed as mean ± standard error (SE). The data shown were from the alanine (50 μM) in day 2 cells, with similar data obtained in the day 9 cells and at other alanine concentrations.

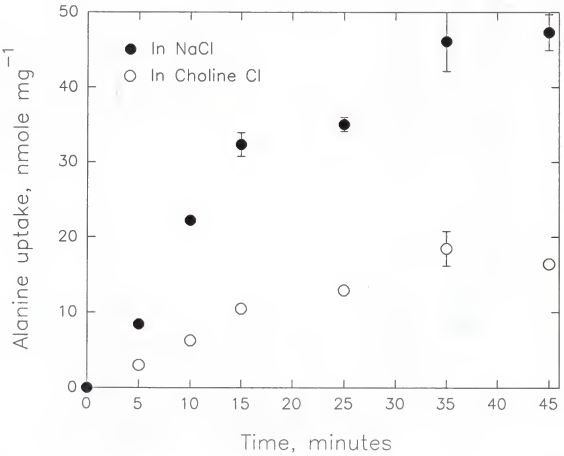

In NaCl
In Choline Cl
Alanine uptake, nmole mg^{-1}
Time, minutes
0
10
20
30
40
50
0
5
10
15
20
25
30
35
40
45

Fig. 3-2. Alanine uptake at various cell ages

The uptake of alanine (50 μM) was measured in NaCl and choline Cl media over cell ages of 1 - 35 days old. At any cell age, the alanine uptake in the NaCl was greater than that in the choline Cl media ($p < 0.05$, $n = 6$), even though the difference margin was smaller in the older cells. The Na^+-dependent alanine uptake decreased with the advancing cell age, while the Na^+-independent alanine uptake also decreased at less extent.

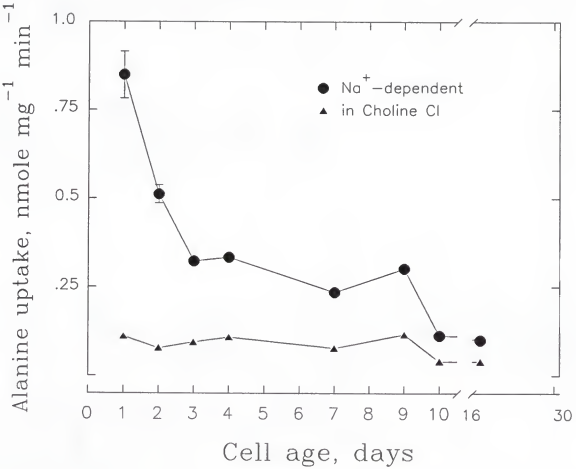

Alanine uptake, nmole mg^{-1} min^{-1}
1.0
.75
0.5
.25
Na^+–dependent
in Choline Cl
0 1 2 3 4 5 6 7 8 9 10 16 30
Cell age, days

Fig. 3-3. Caco-2 cell proliferation rates at various cell ages

The 24 hour incorporation rates of [^{3}H]-thymidine into various cell ages were measured. The cells had been incubated in serum-free DMEM for 24 hours prior to the measurements. The blank control value = 1012 CPM, and the incubation medium value = 3.74 x 10^5 CPM.

Thymidine 10^4 CPM (mg protein)$^{-1}$
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
Cell age, days

Fig. 3-4. Alanine uptake ion-dependency

The uptake of alanine (50μM) was measured in uptake media containing 137 mM NaCl, 137 mM choline Cl, 137 mM KCl, or LiCl. The uptake in NaCl media in both the day 3 and day 8 cells was greater than that in either those in choline Cl, KCl, or LiCl media ($p < 0.05$, $n = 6$). Uptake in choline Cl, KCl, or LiCl media was not significantly different ($p > 0.05$, $n = 6$).

Alanine uptake nmol mg^{-1} min^{-1}
0
0.1
0.2
0.3
0.4
0.5
Day 3
Day 8
CHO
Li$^+$
K$^+$
Na$^+$
*
CHO
Li$^+$
K$^+$
Na$^+$
*

Fig. 3-5. The effect of pH on alanine uptake

The uptake of alanine (50μM) in day 3 and day 8 cells was measured at various medium pH (at pH = 6.1, 7.4, and 8.4). The uptake rates were higher in more alkaline media.

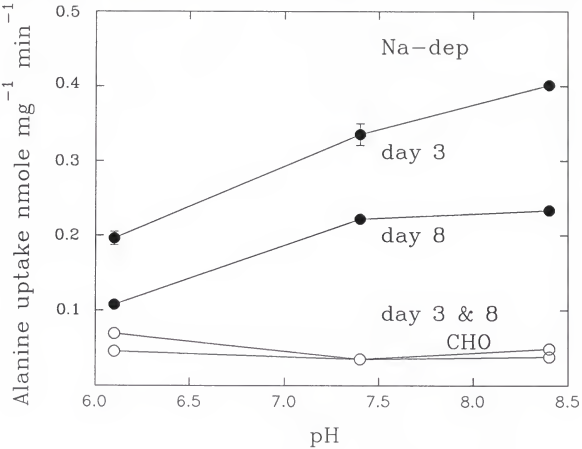

Alanine uptake nmole mg^{-1} min^{-1}
0.5
0.4
0.3
0.2
0.1
Na−dep
day 3
day 8
day 3 & 8
CHO
6.0
6.5
7.0
7.5
8.0
8.5
pH

Fig. 3-6. Na^+-activation of alanine uptake

The uptake of total alanine (50 μM) in day 2 and day 9 cells was measured in media containing various NaCl concentrations ([NaCl] = 0 - 137 mM, choline Cl substituted NaCl). The Non-linear regression of these data gave the same Na^+-activation Hill coefficient of n = 1 for both the day 2 and day 9 cells.

Alanine uptake, nmole mg^{-1} min^{-1}
0.0
0.2
0.4
0.6
0
20
40
60
80
100
120
140
[Na], mM
Day 2
Day 9

Fig. 3-7. Alanine uptake kinetics in day 2 cells

The alanine (10 μM - 5 mM) uptake was measured in the day 2 cells. The figure showed the total alanine uptake rates in NaCl, choline Cl media, or Na^+-dependent alanine uptake rate as a function of alanine concentrations. The curve contained non-saturable and saturable components.

Alanine uptake, nmole mg⁻¹ min⁻¹
[Alanine], μM
Na⁺-dependent
total in CHO
total in NaCl
0
1
2
3
4
5
6
7
8
0
100
200
300
400
500
600
700
800
900
1000

Fig. 3-8. Alanine uptake kinetics in day 9 cells

The uptake of alanine (10 μM - 5 mM) was measured in NaCl and choline Cl media. The total uptake in NaCl and choline Cl media, and Na^+-dependent uptake were showed as a functional of alanine concentrations. The curves showed non-saturable and saturable components.

Alanine uptake, nmole mg^{-1} min^{-1}
1.5
1.0
0.5
0.0
0
100
200
300
400
500
600
700
800
900
1000
[Alanine], μM
Na^+–dependent
total in CHO
total in NaCl

Fig. 3-9. Eadie-Hofstee transformation of Na^+-dependent alanine uptake kinetics in day 2 and day 9 cells

The Na^+-dependent alanine (10 μM - 5 mM) uptake of fig. 3-7 was expressed as alanine uptake as a function of alanine uptake/alanine concentration. Non-linear regression of these data gave a straight line, indicating a single transport system. The V_{max} values (the interception of the line and the y axis) were V_{max} = 3.1 ± 0.21 nmole/mg/min for day 2 cells, and V_{max} = 0.51 nmole/mg/min for day 9 cells. The K_m values (the negative slope of the line) were K_m = 167 ± 26.1 μmole alanine for day 2 cells, and K_m = 159.0 ± 13.6 μmole alanine for day 9 cells.

5.0
4.0
3.0
2.0
1.0
0.0
0
5
10
15
20
○ day 2
□ day 9
J_{ala}, nmole mg^{-1} min^{-1}
J/[Ala], μLiter mg^{-1} min^{-1}

Fig. 3-10. Na^+-independent alanine uptake inhibition pattern in day 3 cells

Alanine (50 μM) uptake in choline Cl medium was measured in day 3 cells, with 5 mM single amino acid present in the uptake media. The Na^+-independent portion was the difference between the total uptake in choline Cl media and passive diffusion.

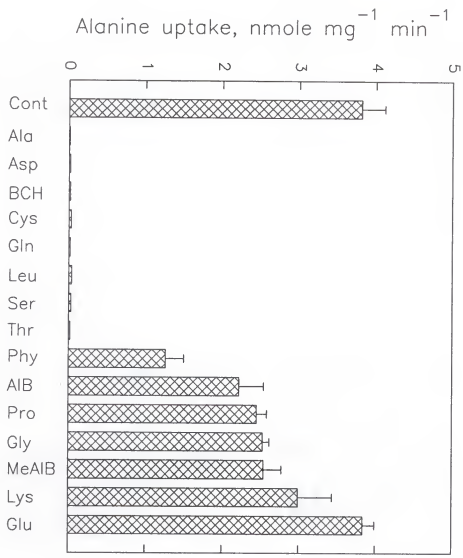

Alanine uptake, nmole mg^{-1} min^{-1}
0
1
2
3
4
5
Cont
Ala
Asp
BCH
Cys
Gln
Leu
Ser
Thr
Phy
AIB
Pro
Gly
MeAIB
Lys
Glu

Fig. 3-11. Na^+-independent alanine uptake inhibition pattern in day 9 cells

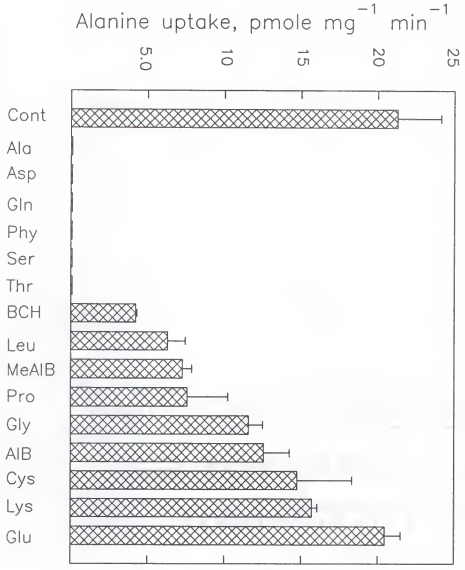

Alanine uptake, pmole mg^{-1} min^{-1}
5.0
10
15
20
25
Cont
Ala
Asp
Gln
Phy
Ser
Thr
BCH
Leu
MeAIB
Pro
Gly
AIB
Cys
Lys
Glu

Fig. 3-12. Na^+-dependent alanine uptake inhibition pattern in day 3 cells

Alanine (50 μM) uptake in day 3 cells was measured in NaCl and choline Cl uptake media containing single 5 mM amino acid. The Na^+-dependent portion was shown.

Alanine uptake, nmole mg^{-1} min^{-1}
0
0.2
0.4
0.6
0.8
1.0
Inhibitors, 5 mM
Control
Cysteine, HCl
Cysteine, free base
Cysteine, free base + DTT
Cysteine, HCl + DTT
Serine
Asparagine
Glutamine
Alanine
Threonine
Methionine
Leucine
Glycine
Tryptophan
Valine
Histidine
Isoleucine
Proline
AIB
Phenylglycine
Arginine
Tyrosine
MeAIB
BCH
DTT
Cystin
Lysine

Fig. 3-13. Dixon analysis of Na^+-dependent alanine uptake with glutamine as inhibitor

Alanine (25 μM, 50 μM, and 100 μM) uptake in NaCl and choline Cl media was measured with various concentration of glutamine (10 μM - 5 mM) presented in uptake media. The dixon plot gave a K_i of 35 μM glutamine.

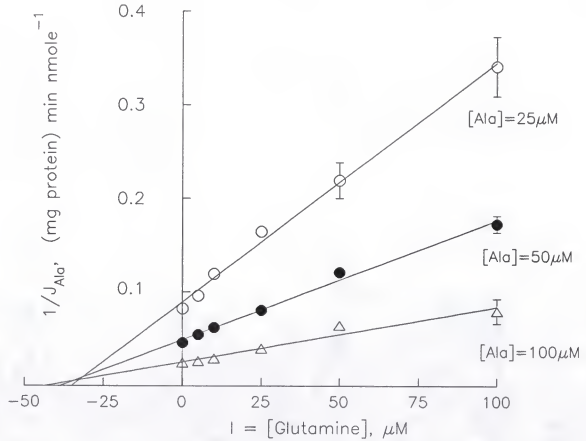

0.4
0.3
0.2
0.1
$1/J_{Ala}$, (mg protein) min nmole^{-1}
[Ala]=25μM
[Ala]=50μM
[Ala]=100μM
−50
−25
0
25
50
75
100
I = [Glutamine], μM

Fig. 3-14. Replot of the slopes of Dixon plot with glutamine as inhibitor

The slopes of dixon plot shown at Fig 3-13 were shown as a function of (corresponding alanine concentrations)$^{-1}$. Non-linear regression of these data intercepted 0. The combination of Fig. 3-13 and this figure indicated that glutamine was a competitive inhibitor for System B.

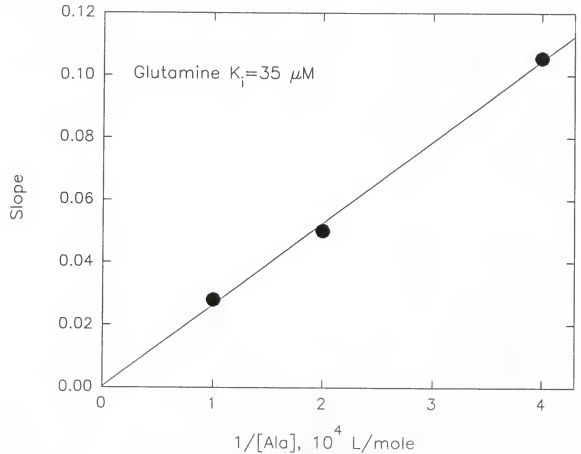

0.12
0.10
0.08
0.06
0.04
0.02
0.00
Slope
Glutamine K_i=35 μM
0
1
2
3
4
1/[Ala], 10^4 L/mole

Fig. 3-15. Dixon analysis of Na^+-dependent alanine uptake with MeAIB as inhibitor

Alanine (25 μM, 50 μM and 100 μM) uptake was measured with various concentrations of MeAIB (10 μM - 5 mM) in uptake media. Non-linear regression of these data were parallel, indicating MeAIB was not a competitive inhibitor.

4.8
3.6
2.4
1.2
[Ala]=25uM
[Ala]=50uM
[Ala]=100uM
−10 −8 −6 −4 −2 0 2 4 6 8 10
[MeAIB], mM
1/J_Ala, (mg protein) min nmole^-1

Fig. 3-16. Dixon analysis of Na^+-dependent alanine uptake with proline as inhibitor

Alanine (25 μM, 50μM, and 100 μM) uptake was measured with various concentrations of proline (10 μM - 5 mM) in uptake media. K_i = 7.1 mM proline.

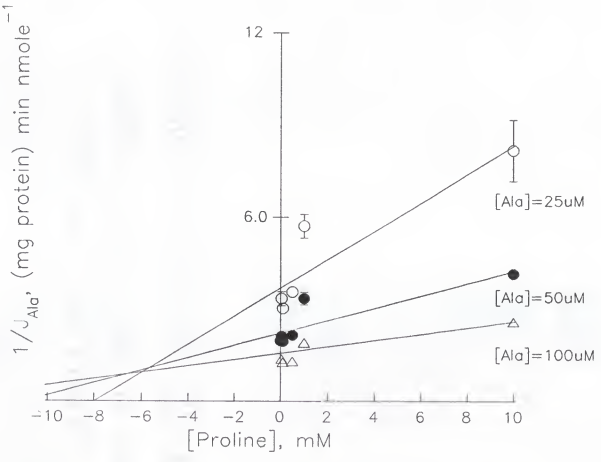

12
6.0
$1/v_{Ala}$, (mg protein) min nmole^{-1}
[Ala]=25uM
[Ala]=50uM
[Ala]=100uM
−10 −8 −6 −4 −2 0 2 4 6 8 10
[Proline], mM

Fig. 3-17. Replot of the slopes of Dixon plot with proline as inhibitor

The slopes of figure 3-16 dixon plot were shown as a function of 1/[alanine]. Non-linear regression of these data was through the interception of x axis and y axis. These data combined with fig. 3-16 suggested that proline was a weak competitive inhibitor for the Na^+-dependent alanine uptake.

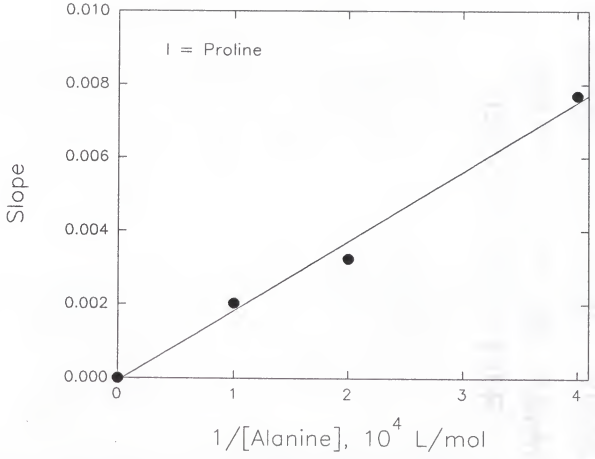

I = Proline
Slope
0.010
0.008
0.006
0.004
0.002
0.000
0
1
2
3
4
1/[Alanine], 10^4 L/mol

Fig. 3-18. Dixon analysis of Na^+-dependent alanine uptake with glycine as inhibitor

Alanine (25 μM, 50 μM, and 100 μM) uptake was measured with various concentrations of glycine (10 μM - 5 mM) in uptake medium. The K_i value was about 5.5 mM glycine.

$1/J_{Ala}$, (mg protein) min nmole^{-1}
15.0
12.0
9.0
6.0
[Ala]=25uM
[Ala]=50uM
[Ala]=100uM
−10 −8 −6 −4 −2 0 2 4 6 8 10
[Glycine], mM

Fig. 3-19. Replot of the slopes of Dixon plot with glycine as inhibitor

The slopes of the dixon plots at Fig. 3-18 were shown as a function of 1/[alanine]. Non-linear regression of these data was through 0 point of both axis. These data combined with fig. 3-18 suggested that glycine was a weak competitive inhibitor of the Na^+-dependent alanine uptake.

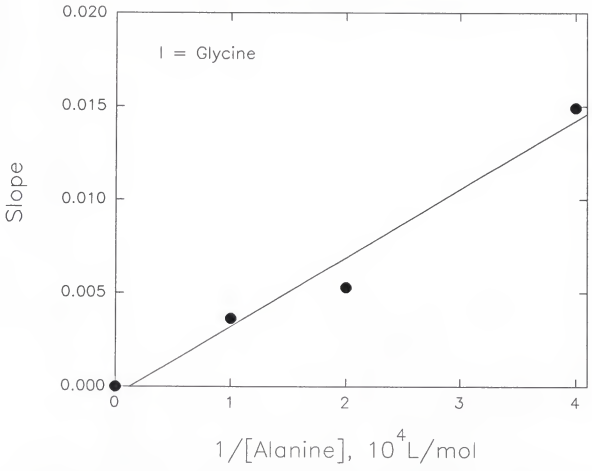

0.020
0.015
0.010
0.005
0.000
Slope
I = Glycine
0
1
2
3
4
1/[Alanine], 10^4 L/mol

Fig. 3-20. Dixon analysis of Na^+-dependent alanine uptake with phenylalanine as inhibitor

Alanine (25 μM, 50 μM, and 100 μM) uptake was measured with various concentrations of phenylalanine in uptake media. Non-linear regression of these data intercepted at x axis, indicating a non-competitive inhibition profile.

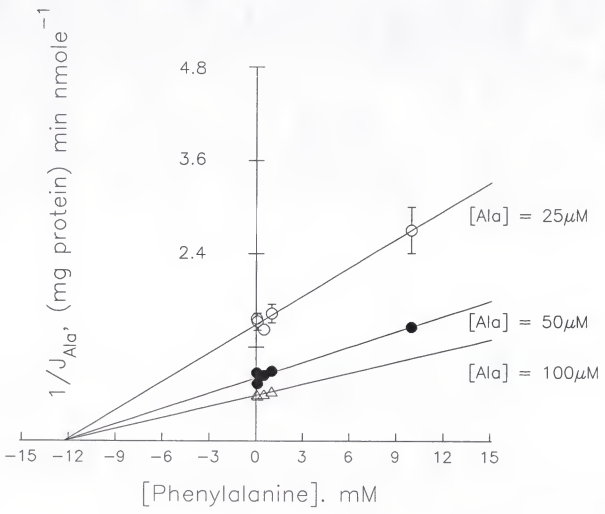

4.8
3.6
2.4
$1/J_{Ala}$, (mg protein) min nmole^{-1}
[Ala] = 25μM
[Ala] = 50μM
[Ala] = 100μM
−15 −12 −9 −6 −3 0 3 6 9 12 15
[Phenylalanine]. mM

Fig. 3-21. Replot of the slopes of Dixon plot with phenylalanine as inhibitor

The slopes of fig. 3-20 were shown as a function of 1/[alanine]. Non-linear regression of these data intercepted at y axis, these data combined with fig. 3-20 suggested that phenylalanine was not a competitive inhibitor of Na^+-dependent alanine uptake.

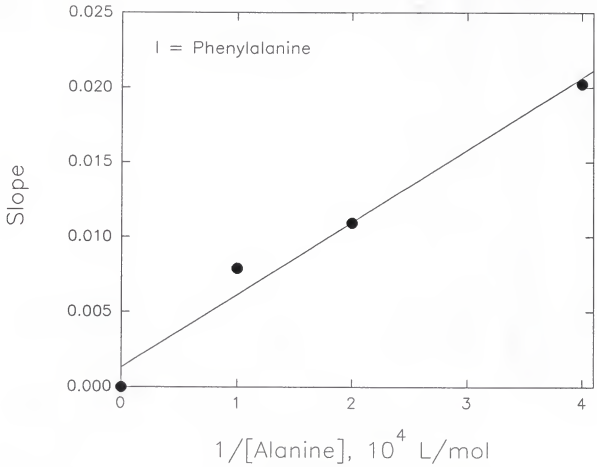

I = Phenylalanine
Slope
0.025
0.020
0.015
0.010
0.005
0.000
0
1
2
3
4
1/[Alanine], 10^4 L/mol

Fig. 3-22. Na^+-dependent alanine uptake inhibition pattern in both day 3 and day 9 cells

Na^+-dependent alanine uptake rates with various 5 mM amino acid in uptake media were measured in both the day 3 and day 9 cells. The uptake in the day 3 cells was shown as a function of the uptake in the day 9 cells. As shown in the figure, the degree of alanine uptake inhibited by amino acids was similar in the day 3 and day 9 cells. Symbol keys: X = MeAIB, B = BCH, J = Cystine, U = AIB, Z = control, A = Ala, C = Cysteine, F = Phe, G = Gly, H = His, I = Ile, L = Leu, M = Met, N = Asn, P = Pro, Q = Gln, R = Arg, S = Ser, T = Thr, V = Val, W = Trp, Y = Tyr.

Alanine uptake, nmole mg^{-1} hr^{-1} (Day 9)
Alanine uptake, nmole mg^{-1} hr^{-1} (day 3)
0
2
4
6
8
0
5
10
15
20
25
TNO
CSA
M
L
G
V
W
I
H
P
B
U
Y
X
J
F
R
Z

Fig. 3-23. Na^+-dependent alanine and Na^+-dependent glucose uptake at various cell ages

Na^+-dependent alanine (50 μM) uptake and Na^+-dependent α-methyl-glucoside uptake (Blais et al., 1987) was shown as a function of Caco-2 cell ages. The alanine uptake decreased, while the glucose uptake increased with advancing cell age. 100% alanine uptake = 0.5 nmole/mg/min; 100% α-methyl-glucoside uptake = 0.12 nmole/mg/min.

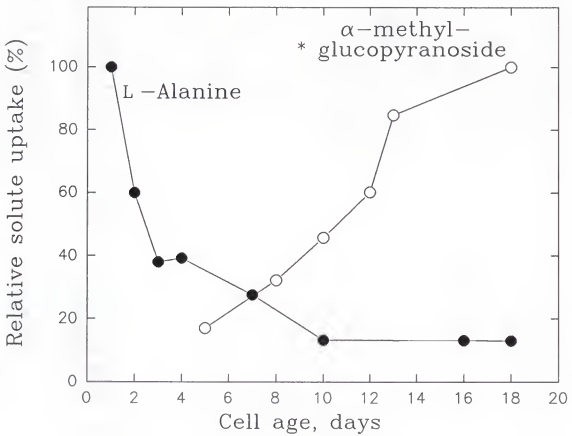

α−methyl−
* glucopyranoside
L −Alanine
Relative solute uptake (%)
0
20
40
60
80
100
0
2
4
6
8
10
12
14
16
18
20
Cell age, days

Fig. 3-24. Apical to basal trans-cellular alanine uptake in cells grown on porous filters

Alanine (50 μM) uptake was measured in cells grown on porous filters. Data shown were the amount of [^{3}H]-alanine transported across cell monolayer from the apical chamber to the basal chamber, in both NaCl and choline Cl uptake media.

Alanine uptake, nmole mg protein^{-1}
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
25
30
Time, minutes
Apical to basal
NaCl
Choline Cl

Fig. 3-25. Alanine apical to basal or apical to cytosol uptake in cells grown on filters

The total amount of 50 μM alanine transported from apical side to basal side, and apical side to cytosol in 30 minute was measured.

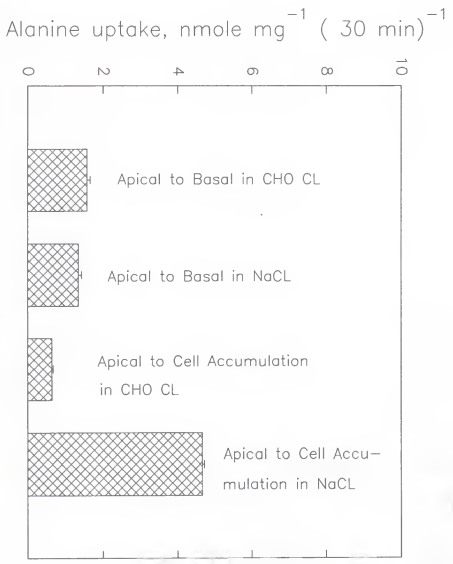

Alanine uptake, nmole mg^{-1} (30 min)$^{-1}$
0
2
4
6
8
10
Apical to Basal in CHO CL
Apical to Basal in NaCL
Apical to Cell Accumulation in CHO CL
Apical to Cell Accu–mulation in NaCL

Fig. 3-26. Alanine uptake across basal membrane in cells grown on porous filters

The total alanine (50 μM) uptake from the basal compartment to cytosol and apical compartments was measured in NaCl and choline Cl media. The amount of basal to cell uptake = the amount of alanine accumulated inside the cells (shown in Fig. 3-26) plus the amount of alanine accumulated in the apical chamber (shown in Fig. 3-27).

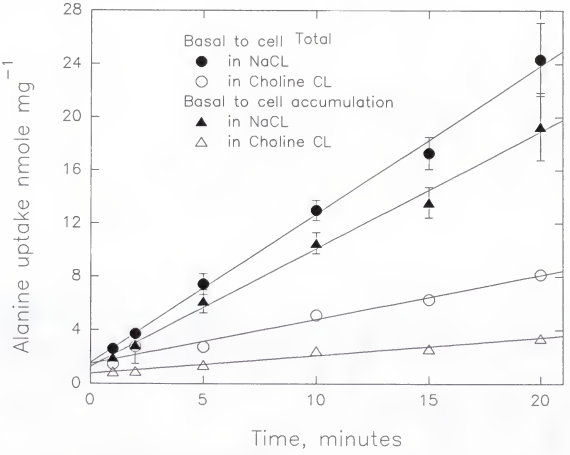

Basal to cell Total
in NaCL
in Choline CL
Basal to cell accumulation
in NaCL
in Choline CL
Alanine uptake nmole mg^{-1}
Time, minutes
0
4
8
12
16
20
24
28
0
5
10
15
20

Fig. 3-27. Alanine basal to cytosol uptake in cells grown on porous filters

Alanine (50 μM) basal to cytosol uptake across basal membrane was measured in NaCl and choline Cl media.

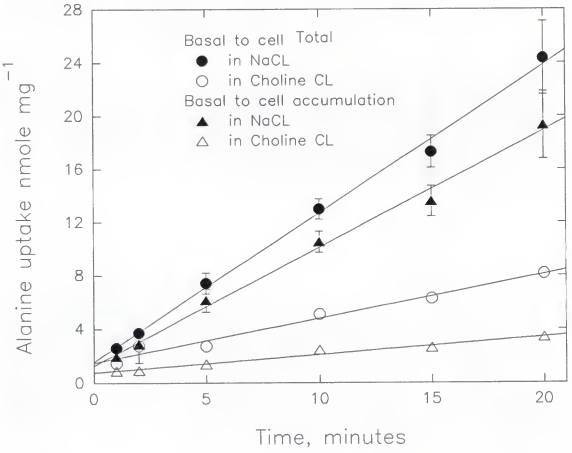

Basal to cell Total
in NaCL
in Choline CL
Basal to cell accumulation
in NaCL
in Choline CL
Alanine uptake nmole mg^{-1}
Time, minutes
0
4
8
12
16
20
24
28
0
5
10
15
20

Fig. 2-28. Alanine basal to apical trans-cellular uptake in cells grown on porous filters

Basal to apical
NaCL
Choline CL
Alanine uptake, nmole mg^{-1}
Time, minutes
0.0
0.5
1.0
1.5
2.0
0
5
10
15
20
25
30

CHAPTER 4
CLASSIFICATION OF THE ARGININE TRANSPORT SYSTEMS IN THE CACO-2 CELL MEMBRANE

4.1 Introduction

The cationic amino acid arginine has attracted much attention during the past years. Not only does arginine possess many important physiological functions, such as blood pressure regulation and urea synthesis, but also its transport systems have unique characteristics. The cloning of system y^+ cDNA marked a breakthrough in the amino acid transport study (Kim et al., 1992). The membrane transport systems for cationic amino acid arginine and lysine have been studied in the past decades, and arginine transport systems has been classified into three systems, a Na^+-dependent System $B^{0,+}$, and two Na^+-independent Systems, y^+ and $b^{0,+}$ (Oxender et al., 1963; Van Winkle et al., 1985, 1987, 1988).

The Na^+-dependent transport System $B^{0,+}$, first described in the blastocyte (Van Winkle et al., 1985) transports both the neutral amino acids and cationic amino acids. System $B^{0,+}$ has not been reported for intestinal cells. The similar System B transporter with characteristics of Na^+-dependent neutral amino acid uptake is exclusively found in intestinal apical membrane, and is considered a variant of System $B^{0,+}$ (Stevens,

1992a,b). The most predominant feature that distinguishes System B from System $B^{0,+}$ is that latter transport arginine and other cationic amino acids.

The Na^+-independent cationic amino acid transport System $b^{0,+}$ has the similar transport characteristics except the Na^+-dependency.

System y^+, originally described in the Ehrlich cell, (Christensen, 1964), reportedly exists in variety of cell types including the intestinal epithelial cells (Christensen, 1975, 1990; Stevens, 1992a,b; Munck, 1981; Hopfer, 1987; White, 1985; Segal et al., 1967; Kilberg et al., 1993). System y^+ Differs from other Na^+-independent neutral amino acid transport Systems L and asc, in that System y^+ has a relative narrow substrate scope selectively serving cationic amino acids. System y^+ activity is not sensitive to pH. Even though System y^+ is a Na^+-independent facilitated transport system, it can transport these cationic substrate against a concentration gradient because of the positive charges possessed by the cationic substrates and the negative electrical PD across the plasma membrane. Unlike System $b^{0,+}$, neutral amino acids does not interact with System y^+ activity in Na-free medium. But in the presence of Na^+, neutral amino acids such as homoserine form a surrogate substrate that can competitively inhibit the System y^+ activity. In the intestinal apical membrane, System y^+ is the predominant transport system for the cationic amino acids transport.

The cloning of system y^+ cDNA has opened a new chapter for membrane amino acid transport study. The discovered system y^+ cDNA also codes for the murine ecotropic retrovirus receptor on cell plasma membrane. System y^+ cDNA was successfully expressed in oocytes. Injection of this cDNA into oocyte results in a increase of typical system y^+ activity (Kim et al., 1991; Wang et al., 1991).

The cDNA encoding NAA/D2, rBAT and 4F2 peptides from rat or rabbit kidney have been expressed in oocytes and apparently increase activity of endogenous System $b^{0,+}$, $B^{0,+}$ activities. These cDNA fragments have been suggested to encode possible regulatory subunits of the system y^+, $b^{0,+}$, or $B^{0,+}$ (Bertran et al., 1992; Magagnin et al., 1992; Tates et al., 1992; Wells et al., 1992a,b).

To examine the arginine transport systems in the Caco-2 cells, we conducted a series of phenomenological studies to define the arginine transport systems. At the time of this study, we did not possess the system cDNA probes for y^+, rBAt, NAA/D2, or 4F2.

4.2 Methods and Materials

4.2.1 Methods

The [^{3}H]-arginine uptake experiments were performed in both the pre-confluent (day 2 - 3) cells and the confluent (day 8 - 9) cells. The basic uptake procedures were as

described in the chapter 2 general methodology section. The uptake experiments with special treatments will be mentioned below where appropriate.

4.3 Results

4.3.1 Arginine Uptake Time Course

The uptakes of 5 μM and 1 mM [^{3}H]-arginine were measured in the Caco-2 monolayer (cell ages day 2 and day 9) at increasing times (0 - 30 minutes) in uptake media containing 137 mM NaCl or 137 mM choline Cl. A representative time course for 5 μM arginine uptake in day 2 cells is shown (Fig. 4-1). During the course of uptake, the [^{3}H]-arginine accumulated inside cells in NaCl medium was not different from that in the choline Cl medium. This indicated that the arginine uptake was mainly a Na^+-independent phenomenon. The initial arginine accumulation in the cells was linear during the initial 10 minutes (at both the [^{3}H]-arginine concentrations of 5 μM and 1 mM). The initial arginine uptake rate that represent the transport activity was obtained by dividing the total arginine accumulation by the uptake time period (within the linear accumulation limit). The uptake period of 0 - 5 minutes was chosen for all the subsequent uptake measurements to ensure the uptake rates represented the true initial arginine transport.

4.3.2 Arginine Uptake Rates Decrease with Caco-2 Cell Age

The 5 μM [^{3}H]-arginine uptake rates in choline medium were measured at various Caco-2 cells age from cell ages of 1 day old to 14 days old. The arginine uptake in the choline Cl uptake media decreased while the cell age increased (Fig. 4-2). The decline in arginine uptake rates was more rapid in the pre-confluent cells than for the older cells. The decrease in arginine uptake was paralleled the decrease in alanine uptake with increasing cell age (Fig. 3-2). As we discussed in Chapter 3, the cell proliferation rates also decreased with advancing cell ages (Fig. 3-3). The arginine uptake change and the alanine uptake change with cell ages may well be due to the decrease of cell proliferation rates at older cells. As discussed below, the passive diffusion coefficient for arginine uptake was the same in day 2 and day 9 cells, suggesting the decrease in arginine uptake with cell age was due to a mechanism other than diffusion.

4.3.3 The Effect of pH on Arginine Uptake

The Na^+-independent arginine uptake was measured in choline Cl medium at pH 6.4 - 8.4 (HEPES/Tris buffers). The arginine uptake in both the day 2 and day 9 cells was unaffected by uptake buffer pH changes (Fig. 4-3).

4.3.4 Arginine Uptake Kinetics

Arginine uptake in the choline Cl media was measured at various arginine concentrations ranging from [arginine] = 0.1 μM to [arginine] = 1 mM for both day 2 and day 9 cells. The uptake kinetics are shown as the uptake rates plotted as a function of arginine concentrations (Fig. 4-4). The kinetics studies of the arginine (concentration range 1 μM - 1 mM) transport in the choline Cl buffer indicated that there was a non-saturable component and Na^+-independent saturable component. The Eadie-Hofstee transformation gave one non-saturable component as passive diffusion and a single Na^+-independent carrier system (Fig. 4-5).

The non-saturable component was simple passive diffusion. The diffusion coefficient for both the day 2 and day 9 cells was the same, P = 1.1 μliter/mg protein/min.

For the single carrier-mediated system, the non-linear regression analyses of the Eadie-Hofstee transformation of the kinetics data gave a V_{max} = 430 pmole/mg protein/min and K_m = 31 μmole arginine for the day 2 cells, while at the day 9 cells the V_{max} = 340 pmole/mg protein/min and K_m = 37 μmole/mg protein/min. These kinetic data suggested that the same transport system was operative at the two cell ages, and the activity difference was a V_{max} effect reflecting a change in the functional transporter units.

4.3.5 Amino Acid Analogue Cross-inhibition

The Na^+-independent arginine uptake activities in choline Cl media which contained single amino acid analogues (5 mM each of the natural amino acids, ornithine, homoserine plus sodium, or D-arginine) were measured at both the day 2 and day 9 cells. The pattern and degree of the amino acid analog inhibition for the Na^+-independent arginine transport was identical in both cell states, suggesting that same transporter system was operative regardless of the cell age (Fig. 4-7). The Na^+-independent [^{3}H]-arginine transport was strongly inhibited only L-lysine, L-arginine, ornithine, and histidine; weaker inhibitors were D-arginine, D-lysine, homoserine (in Na^+ buffer), tryptophan, and methionine; the amino acids which inhibited less than 20 % arginine uptake included alanine, BCH, phenylalanine threonine, serine, asparagine, valine, homoserine (in choline Cl media) and leucine (Fig. 4-6). The analogue cross-inhibition patterns were consistent with that of system y^+ (White, 1985). Dixon analysis of [^{3}H]-arginine uptake inhibited by ornithine, homoserine, and D-arginine revealed that ornithine was a classic competitive inhibitor, while the homoserine was a weaker inhibitor, and D-arginine showed an uncompetitive weak inhibition effects (Fig. 4-8 to 4-12).

4.3.6 Arginine Uptake in Caco-2 Cells on Porous Filters

Trans-epithelial arginine (5 μM) uptake was measured from apical to basal sides in confluent Caco-2 cells (14 days old). The intact confluent monolayers with trans-cellular resistance $\geq$ 300 $\Omega \cdot cm^2$ were used. The arginine trans-cellular rate from apical side to basal side was steady during a 30 minute period (Fig. 4-13). Cellular accumulation of arginine during 30 minutes was 5 times greater than the apical to basal trans-cellular movement (Fig. 4-14).

Arginine (5 μM) movement from basal side to cellular and apical side was also measured in intact confluent Caco-2 cell monolayers. The basal-apical side transport rate was steady for up to 30 minutes (Fig. 4-15). During a 30 minute incubation, the [^{3}H]-arginine trapped in the cellular compartment was 5 times greater than amount of arginine across basal-apical compartment (Fig. 4-16).

4.4 Discussion

The arginine uptake activity across Caco-2 cell monolayer was studied at various cell stages of development. By using classic transport system criteria, we classified the arginine transport systems in the Caco-2 in the undifferentiated state (day 2) and differentiated state (day 9).

4.4.1 The Decrease in Arginine Uptake Activity as Cells Age

The Caco-2 total arginine uptake rates in choline Cl medium decreased as the cells aged (Fig. 4-2). Furthermore, the arginine passive permeability diffusion coefficient in both the undifferentiated day 2 cells and differentiated day 9 cells was the same. The decrease in transport activity with cell age was therefore due to the non-diffusion portion of uptake. In the light of the reduced proliferation rates characteristic of the older cells, the decrease in System y^+ activity with the cell age increase may be associated with the cell's reduced requirement for amino acids.

4.4.2 Classification of Arginine Transport Systems

The kinetic data (Fig. 4-4 & Fig. 4-5) indicated that there was Na^+-independent transport system plus simple passive diffusion at both cell differentiation stages.

The simple passive diffusion coefficients were constant at different cell ages, suggesting that the cell aging was not associated with the diffusion changes.

The amino acid analogue inhibition pattern of the carrier-mediated arginine uptake was strongly inhibited only by lysine, ornithine, and histidine. Homoserine in choline Cl medium has weak inhibition effects, but its inhibition effect was enhanced by the presence of NaCl in the uptake media. The neutral amino acids alanine, phenylalanine, leucine did not

inhibit the arginine uptake in the choline Cl media, consistent with the cross inhibition pattern described for System y^+. The kinetics and pH insensitivity, combined with the inhibition patterns together strongly indicated that the Na^+-independent carrier-mediated arginine transport system in Caco-2 cells was System y^+.

System $b^{0,+}$ is another Na^+-independent transporter of arginine, and is the counterpart of the system $B^{0,+}$. The weak inhibition of arginine uptake by the neutral amino acids alanine and leucine indicated the an unlikely major involvement of system $b^{0,+}$ in the current passages of our Caco-2 cells (passages # 18 - 50). The non-inhibition effect of alanine, BCH, Phenylalanine, and leucine excludes the possible involvement of systems L, or asc.

Over the arginine concentration of 1 μM to 1 mM, the arginine uptake in NaCl medium was not different from that in the choline Cl. These data suggested that the arginine uptake in the Caco-2 cells was mainly a diffusion plus Na^+-independent system y^+ transport event; no Na^+-dependent transport phenomenon is involved.

The Na^+-independent carrier-mediated system, affinity characteristics, inhibition patterns, and pH insensitivity were the same in both the undifferentiated day 2 and differentiated day 9 cells. Only the V_{max} value was higher in day 2 cells compared to day 9 cells (Figs. 4-3 - 4-12). These combined data indicated that arginine was transported through

the same System y^+ in both the day 2 and day 9 cells. The kinetic parameters (Fig. 4-5) strongly suggested that the change in transport capacity during cell development was likely due to the number of copies of functioning transport units in the apical membrane (per cell mass), rather than modification of characteristics of existing transporters.

The arginine uptake measurements in the Caco-2 monolayer grown on the porous filters showed that the majority of uptaked arginine accumulated inside the cells. Arginine exits from the cytosol across either the basal membrane or apical membrane to the extracellular media at a much slow rate than the arginine transport across the membrane from outside to inside rates.

4.5 Summary

Arginine is transported in Caco-2 cells by passive diffusion and System y^+. System y^+ behaves with the same kinetic characteristics operative in both the undifferentiated and differentiated states. The System y^+ capacity is down-regulated during the cell development, while the diffusion coefficient is not affected. The system y^+ activity decrease is coincident with the declining cell proliferation rate. The decrease in System y^+ activity is likely caused by the decrease of number of copies of functional transporter units, rather than the modification of existing transport affinity for substrate.

Fig. 4-1. Arginine uptake time course

The uptake of arginine (5 μM and 1 mM) was measured in choline Cl and NaCl uptake media in the day 2 and day 9 cells. The total arginine accumulation inside the cells was measured at various time periods (0 - 30 minutes). At each point, the total arginine uptake in choline Cl medium was > 90% of those in the NaCl medium. The data shown was from the arginine (5 μM) uptake in day 2 cells, similar results were obtained in other cell ages (day 9) and other arginine concentrations (1 mM). Na^+-independent pathway was the major uptake mechanism in Caco-2 cells.

L-Arg uptake, nmole mg^{-1}
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
30
Time, minutes
in Na
in CHO

Fig. 4-2. Arginine uptake at various cell ages

Arginine (5 μM) uptake was measured in choline Cl uptake medium over the cell ages of 2 - 14 days old. The uptake rates decreased as cell age increased, with rapid decrease in the pre-confluent cells (< 6 days old).

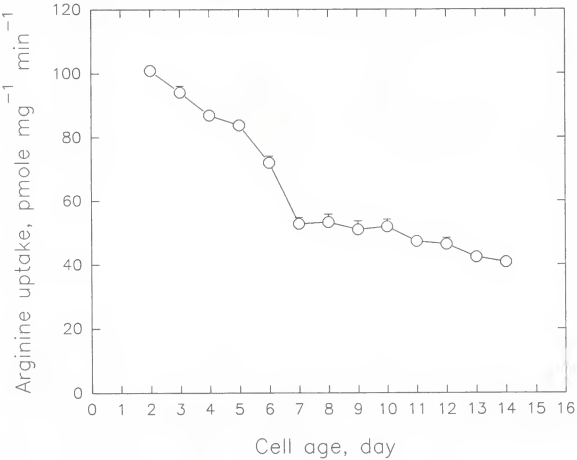

Arginine uptake, pmole mg^{-1} min^{-1}
120
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Cell age, day

Fig. 4-3. The effect of pH on arginine uptake

Arginine (5 μM) uptake in day 2 and day 9 cells was measured in choline Cl medium, with various medium pH (pH 6.1, 7.4, or 8.4). The pH was adjusted by using 10 mM HEPES and 10 mM Tris buffer. The arginine uptake was not affected by the medium pH.

Arginine uptake, pmole mg⁻¹ min⁻¹
150
120
90
60
30
6.0
6.6
7.2
7.8
8.4
pH
day 9 cells
day 2 cells

Fig. 4-4. Arginine uptake kinetics

Arginine (0.1 μM - 1 mM) uptake was measured in choline Cl medium in day 2 and day 9 cells. The total uptake rates at each arginine concentration were showed as a function of arginine concentration. The shape of the kinetic curves indicated the existence of both non-saturable and saturable components.

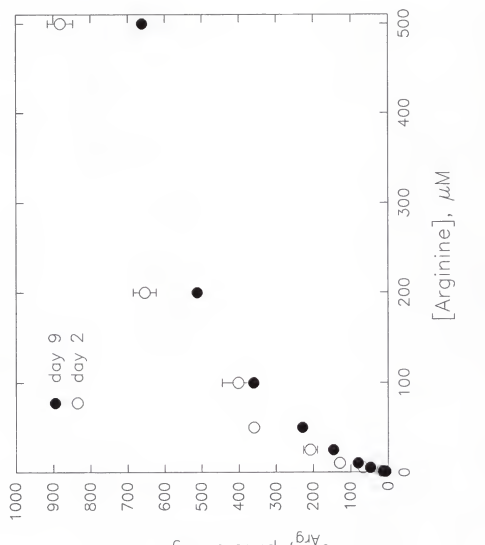

1000
900
800
700
600
500
400
300
200
100
0
0
100
200
300
400
500
day 9
day 2
J_{Arg}, pmole mg^{-1} min^{-1}
[Arginine], μM

Fig. 4-5. Eadie-Hofstee transformation of Na^+-independent arginine uptake kinetics in day 2 and day 9 cells

The arginine (0.1 μM - 1 mM) uptake rates described in the fig. 4-4 was showed as a function of $J_{arg}/[arg]$. The Na^+-independent portion was the difference of total arginine uptake in choline Cl medium and the passive diffusion at the [arginine]. Non-linear regression of the data gave straight lines, indicating a single transport system in each of these day cells. V_{max} was 430 pmole/mg/ml in day 2 cells, and V_{max} = 340 pmole in day 9 cells. The K_m values were K_m = 31 μmole arginine for day 2 cells, and K_m = 37 μmole arginine in day 9 cells.

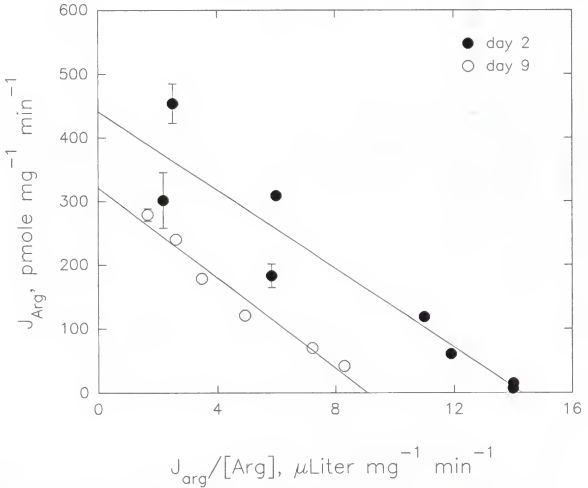

600
500
400
300
200
100
0
0
4
8
12
16
day 2
day 9
J_{Arg}, pmole mg^{-1} min^{-1}
$J_{arg}/[Arg]$, μLiter mg^{-1} min^{-1}

Fig. 4-6. Arginine Na^+-independent uptake inhibition pattern in day 3 cells

Arginine (5 μM) uptake in choline Cl medium was measured in day 3 cells, with 5 mM single amino acid present in the uptake medium. Similar results were obtained in day 9 cells.

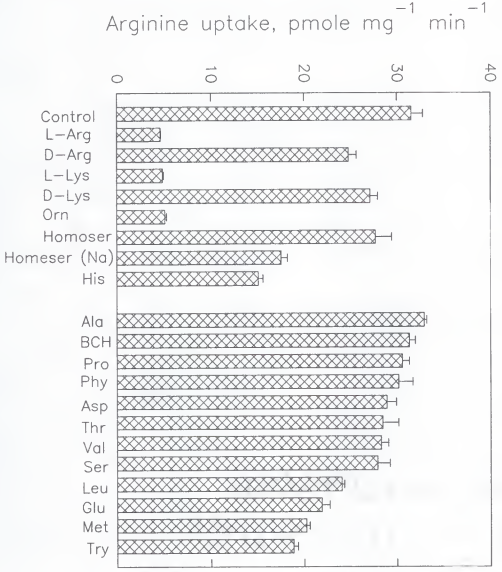
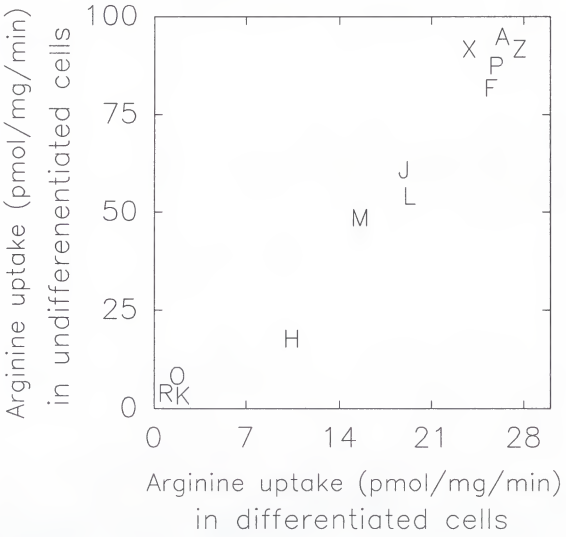

Arginine uptake, pmole mg^{-1} min^{-1}
0
10
20
30
40
Control
L−Arg
D−Arg
L−Lys
D−Lys
Orn
Homoser
Homeser (Na)
His
Ala
BCH
Pro
Phy
Asp
Thr
Val
Ser
Leu
Glu
Met
Try

Fig. 4-7. Arginine Na^+-independent uptake inhibition pattern in day 3 and day 9 cells

Na^+-independent arginine (5 μM) uptake rates with 5 mM amino acid in uptake media were measured in both the day 3 and day 9 cells. The uptake rates in the day 3 cells were shown as a function of the uptake rates in the day 9 cells. The degree of arginine uptake inhibited by amino acids was similar in both cell ages.



Competitive Inhibitors

X MeAIB
Z control
J Homoserine plus Na
R Arg
K Lys
O Ornithine
A Ala
F Phe
H His
L Leu
M Met
P Pro

Fig 4-8. Dixon analysis of Na^+-independent arginine uptake with ornithine as inhibitor

Arginine (o.5 μM, 5 μM, and 50 μM) uptake was measured with various concentration of ornithine (1 μM - 1 mM) in choline uptake medium. Non-linear regression gave a K_i = 80 μM ornithine.

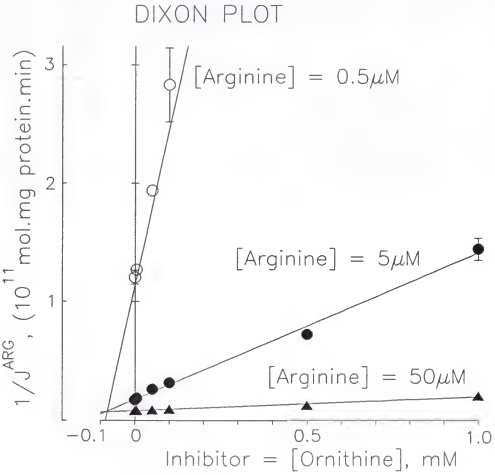

DIXON PLOT
[Arginine] = 0.5μM
[Arginine] = 5μM
[Arginine] = 50μM
1/J^ARG, (10^11 mol.mg protein.min)
3
2
1
−0.1
0
0.5
1.0
Inhibitor = [Ornithine], mM

Fig. 4-9. Replot of the slopes of Dixon plot with ornithine as inhibitor

The slopes of fig. 4-8 were shown as a function of 1[arginine]. Non-linear regression of these data was through 0 of the axis, indicating that ornithine was a competitive inhibitor of the arginine uptake.

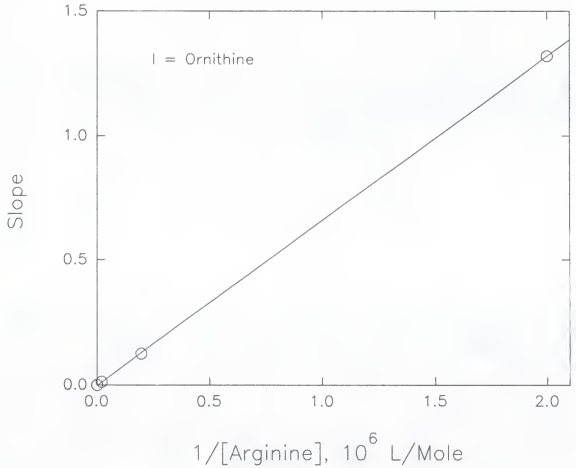

I = Ornithine
Slope
1.5
1.0
0.5
0.0
0.0
0.5
1.0
1.5
2.0
1/[Arginine], 10^6 L/Mole

Fig. 4-10. Dixon analysis of Na^+-independent arginine uptake with homoserine as inhibitor

Arginine (0.5 μM, 5 μM, and 50 μM) uptake was measured with various concentration of homoserine (1 μM -1 mM) in choline Cl uptake medium. Non-linear regression of these data gave a K_i of 570 μM for homoserine.

0.5
0.4
0.3
0.2
−1.0
−0.5
0.0
0.5
1.0
[Arg]=0.5μM
[Arg]=5μM
[Homoserine], mM
1/J_arg, 10^12 mg/min/mole

Fig. 4-11. Dixon analysis of Na^+-independent L-arginine uptake with D-arginine as inhibitor

Arginine (0.5 μM, 5 μM, and 50 μM) uptake was measured with various concentrations of D-arginine in choline Cl uptake medium. Non-linear regression of these arginine uptake indicated that D-arginine was not inhibitor of arginine uptake.

0.20
0.15
0.10
0.05
0.00
-0.05
0.0
0.2
0.4
0.6
0.8
1.0
[D-Arginine], mM
[L-Arg] = 0.5 μM
[L-Arg] = 5 μM
[L-Arg] = 50 μM
1/J_Arg, mg min pmole^-1

Fig. 4-12. Arginine apical to basal trans-cellular uptake in cells grown on porous filters

Arginine (5 μM) uptake from the apical chamber to the basal chamber was measured in choline Cl medium ± 0.5 mM L-lysine as inhibitor.

Apical to Basal
total in Choline CL
in Choline CL (+0.5 mM Lys)
Arginine uptake, nmole mg^{-1}
Time, minutes
0.0
0.2
0.4
0.6
0.8
1.0
0
5
10
15
20
25
30

Fig. 4-13. Arginine apical to cytosol and apical to basal uptake in cells grown on porous filters

The arginine (5 μM) movement from the apical side to cytosol and apical to basal in 30 minutes was measured.

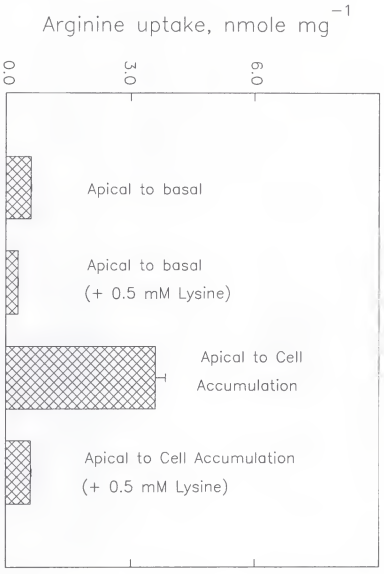

Arginine uptake, nmole mg^{-1}
0.0
3.0
6.0
Apical to basal
Apical to basal
(+ 0.5 mM Lysine)
Apical to Cell
Accumulation
Apical to Cell Accumulation
(+ 0.5 mM Lysine)

Fig. 4-14. Arginine basal to apical trans-cellular uptake

Arginine (5 μM) uptake from the basal side to the apical side was measured at various time periods.

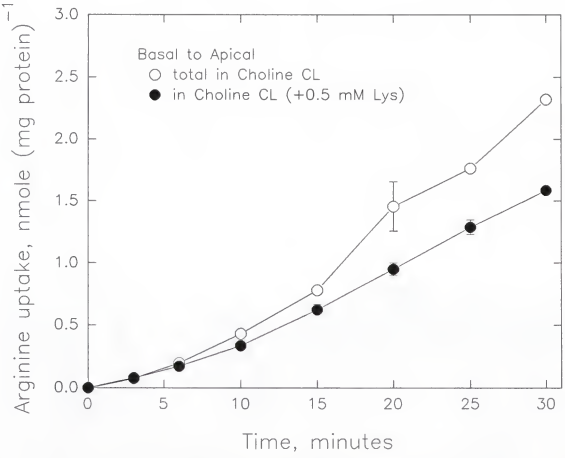
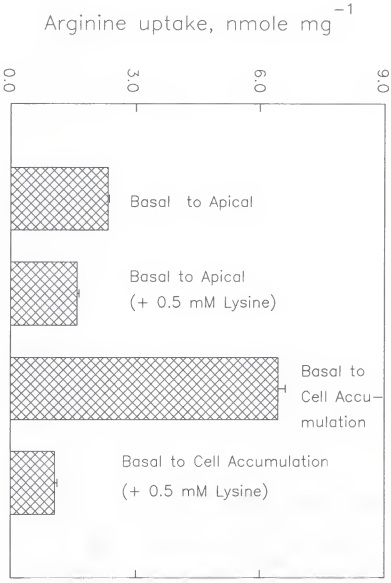

Basal to Apical
total in Choline CL
in Choline CL (+0.5 mM Lys)
Arginine uptake, nmole (mg protein)$^{-1}$
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
25
30
Time, minutes

Fig. 4-15. Arginine basal to cytosol and basal to apical uptake

The arginine (5 μM) uptake (30 minutes) at basal side in choline Cl medium ± 0. 5 mM lysine was measured. Data shown were the total arginine in the apical side and inside the cells at 30 minute uptake.


Arginine uptake, nmole mg^{-1}
0.0
3.0
6.0
9.0
Basal to Apical
Basal to Apical
(+ 0.5 mM Lysine)
Basal to
Cell Accu-
mulation
Basal to Cell Accumulation
(+ 0.5 mM Lysine)

CHAPTER 5
THE EFFECT OF INDIVIDUAL AMINO ACIDS ON SYSTEM B AND SYSTEM y^+ TRANSPORT ACTIVITIES

5.1 Introduction

Intestinal epithelial cells encounter various amino acids concentrations in the lumen at various times. The luminal concentrations of amino acids depend on the timing of food intake and on food composition. In contrast to the cells of many internal organs, the small intestine adaptively up-regulates its amino acid transport capacity in response to the increase of amino acids concentrations exposed (Stevens, 1991, 1992a,b; Salloum et al., 1990; Scharrer et al., 1981; Stein et al., 1987; Ferraris et al., 1988a,b; Diamond & Karasov, 1987; Ferraris & Diamond, 1989).

The transport activity of system A in the hepatocytes and MDCK cells increased upon prolonged amino acid starvation. Addition of amino acids to the amino acid deficient medium resulted in a loss of system A activity (Kilberg et al., 1985; Boerner & Saier, 1985; Bracy et al., 1985). Substrate regulation of the system A activity involves a short-term cycloheximide-insensitive mechanism and a long-term cycloheximide-sensitive mechanism (Boerner & Saier, 1985). Whether the short-term inhibition is involved a trans-

inhibition mechanism or other regulatory mechanism is still inconclusive. The long-term regulation involves a transcriptional and translational regulatory mechanism. Two regulatory mechanisms have proposed to explain the system A substrate regulation (Kilberg, 1986; Englesberg et al, 1986).

Substrate regulation of amino acid transport systems of intestinal epithelial cells ha been investigated in whole animals fed various diets (Ferraris & Diamond, 1989; Ferraris et al., 1988a,b). Animal feeds with high protein or high amino acid diet increased their transport of non-essential amino acids, such as aspartate. Essential amino acid transport activity decreased in animals on high amino acid diets. In general, the transporter substrates are often the best inducers of uptake capacity, but sometimes the best inducers are un-related to the transport systems (Stein et al., 1987; Diamond & Karasov, 1987; Scharrer et al., 1981). The up-regulation of the intestinal amino acids uptake occurs over 0.5 - 1 day, whereas the reversal of the up-regulated level to the original level takes several days.

The mechanism of intestinal substrate induction of transport has not been addressed at cellular level. In this study, we explored the effect of individual amino acids to increase the activity of System B and System y^+ in both short-term and long-term exposures.

5.2 Methods and Materials

Caco-2 cells were incubated in depletion medium for 2 hours, and then incubated in the same medium containing various concentration of alanine, arginine, or other amino acids ± cycloheximide (10 μM or 50 μM) for various windows of time (30 seconds up to 48 hours) in the 37°C incubator. The incubation medium was changed every 8 hours to ensure that amino acid concentration were constant, and to remove possible build up of autocrine.

The depletion medium contained 0.265 g/L $CaCl_2$, 0.0001 g/L Ferric Nitrate, 0.09767 g/L $MgSO_4$, 0.4 g/l $NaHCO_3$, 6.4 g/L NaCl, 0.109 g/L NaH_2PO_4, 4.5 g/L glucose, 0.0159 g/L phenol Red·Na, 0.004 g/L choline chloride, 0.004 g/L folic acid, 0.0072 g/L myo-inositol, 0.004 g/L niacinamide, 0.004 g/L D-pantothenic acid, 0.004 g/L pyridoxa·HCl, 0.0004 g/L riboflavin, and 0.004 g/L thiamine·HCl.

5.3 Results

5.3.1 System B Activity Decrease in Starved Caco-2 Cells

The system B alanine uptake rate declined as early as 15 minutes following depletion medium incubation, and reached the lowest level at 2 hours, where they remained steady for at least 48 hours. The system B alanine uptake rate in the Caco-2 cells which were incubated in depletion medium plus 1 mM

alanine also decreased as incubation progress, however, the decrease was less than that in cells incubated in depletion medium alone. The 50 μM alanine System B uptake rate in the alanine-incubated cells was 50% higher than that in the depletion-incubated cells (Fig. 5-1).

5.3.2 System B Activity was Activated by Acute Amino Acid Exposure

The Caco-2 cells were incubated in the depletion medium containing 1 mM individual amino acids for 3 hours. Mannitol (1 mM) was the control. Cysteine solutions contained 1 mM dithiothreitol, with the 1 mM dithiothreitol solution serving as control. System B activities were measured immediately after each incubation. In comparison to the cells incubated in the control depletion medium alone, the increase in System B activity in cells incubated in solution containing amino acids gave a pattern of stimulation that matched the ranking of System B substrates (described in the chapter 3). That was, alanine, serine, glutamine, threonine and cysteine each increased the system B activity by 1.5 - 2 fold; the weaker stimuli (which weakly inhibited system B activity in the cross-inhibition study (Fig. 3-12)) were amino acids such as histidine, glycine, and valine. Finally phenylalanine, leucine, lysine, arginine, and MeAIB, which did not inhibit system B activity in cross-inhibition study, did not stimulated the system B activity (Fig. 5-2).

In a separate study, 1 mM alanine was added to Caco-2 cells which had been incubated in the depletion medium for 3 hours. Within 5 minutes the system B activity was increased compared to the control level. After 2 hours in depletion incubation, cells were incubated in the alanine metabolism inhibitor aminooxy acetic acid (AOA, 2.5 mM). The system B alanine uptake rate increased only of 7 hours AOA incubation (Fig. 5-3).

MeAIB uptake was measured in Caco-2 cells which had been incubated in depletion medium, ± 1 mM alanine, 1 mM serine or 1 mM MeAIB for 3 hours. The MeAIB (2.5×10^{-8} M) uptake was not affected by the incubation with added 1 mM alanine or serine. MeAIB incubation decreased the MeAIB uptake (Fig. 5-6).

The Na^+-independent system L alanine uptake was also measured in above experiment conditions. The system L alanine uptake was increased by alanine, serine, glycine, and was not affected by MeAIB and proline.

5.3.3 Short-Term Activation of System B Activity did not Involve Protein-Synthesis

The Caco-2 cells were washed three times with the depletion medium, and incubated with the same medium containing 1 mM alanine ± 50 μM cycloheximide for 3 hours. The 50 μM alanine system B activity was measured. The system B alanine uptake was increased 2 folds by the alanine incubation alone. The cycloheximide alone in the incubation

medium did not affect the alanine uptake, nor did the CHX in the alanine incubation medium affect the increase of alanine uptake (Fig. 5-4).

5.3.4 The System B Activity Increase by Substrate Acute Exposure was Reversible

The Caco-2 cell were washed and incubated in the depletion medium containing 1 mM alanine (mannitol as control) for 3 hours as described above. The cells were then washed three times with the depletion medium, and incubated in the depletion medium (lacking amino acids) for 3 hours. The 50 μM alanine system B activity was increased 2 fold after incubation for 3 hours with alanine, and returned to the control level after the additional 3 hours in alanine-free depletion medium incubation.

5.3.5 The System B Activity Increased by its Substrate Exposure Involved Kinetic Modifications

The Caco-2 cells were washed three time with the depletion medium, and incubated in the same medium ± 1 mM alanine or DMEM for 3 hours. The kinetics of the system B transport activity ([^{3}H]-alanine concentration = 1 μM - 5 mM) showed that the alanine incubation resulted in a 2 fold V_{max} increase and plus an increase of K_m (Fig. 5-5). The DMEM incubation also resulted in a increase of V_{max} and K_m. These data indicated that the system B activity increase is likely involved in the modification of the transport system affinity

to bind alanine and/or Na^+

5.3.6 System B Activity Increased by Chronic Alanine Exposure was Dependent on Protein Synthesis and PKC activation

The Caco-2 cells were washed with the depletion medium, and incubated in the same medium containing 0, 0.1, 1, or 10 mM alanine, ± 10 μM cycloheximide or 6.6 μM chelerythrine Cl for 24 hours. The medium was changed every 6 hours. The 50 μM alanine system B activity was then measured. The system B activity was increased by various alanine exposures, with greater alanine concentrations causing a greater stimulation effect. The alanine stimulation was partially blocked by cycloheximide or chelerythrine (Fig. 5-7 & Fig. 5-8).

Caco-2 cells were then pre-incubated with alanine for 24 hours, as described above, and the cells were then incubated in the depletion medium for 3 hours before System B activity measurement. System B activity was increased by exposure to alanine, and this increase was then completely blocked by cycloheximide or chelerythrine.

5.3.7 System y^+ Activity Decreased in Starved Caco-2 Cells

The Caco-2 cells were washed three times with the depletion medium, and then incubated in the same medium ± 1 mM L-arginine or D-arginine for various length of time (30 seconds to 48 hours) in the 37°C incubator. One millimole mannitol was used as control. The medium was changed every 6

hours to ensure that the amino acid concentration was constant and the possible autocrine accumulation was eliminated. The system y^+ transport activities were measured immediately after each incubation period. The 5 μM arginine system y^+ uptake decreased as the incubation time increased, and reached the lowest level at about 3 hours where they stayed 48 hours. The declining system y^+ activity was partially prevented by exposure to L-arginine or D-arginine (at lesser degree) (Fig. 5-9). The 3 hours depletion incubation was chosen for the subsequent experiments.

5.3.8 System y^+ Activity was Stimulated by Acute Amino Acid Exposure

The Caco-2 cells were washed three time with the depletion medium, and incubated in the same medium containing 1 mM individual amino acids (1 mM mannitol as control, all cysteine solution also contained 1 mM dithiothreitol in the case 1 mM dithiothreitol was used as control) for 3 hours. The System y^+ activity was measured immediately after each incubation. The System y^+ activity was increased two-fold by the system y^+ substrates lysine, arginine, ornithine. System y+ non-substrates proline, BCH, and alanine did not affect the System y^+ activity . A pattern emerged such that these amino acids which weakly inhibited System y^+ activity also weakly stimulated System y^+ activity (Fig. 5-10). These data suggested that the system y^+ activity was specifically

stimulated by its own substrates.

In another study, Caco-2 cells were incubated in the depletion medium for 3 hours, and 1 mM arginine was added to the medium. System y^+ arginine uptake increased as early as 5 minutes arginine incubation.

5.3.9 System y^+ Activity Increased by Acute Arginine Exposure did not Involve Protein Synthesis

The Caco-2 cells were washed and incubated in the depletion medium containing 1 mM arginine (1 mM mannitol as control) ± 50 μM cycloheximide or 0.5 μg/ml actinomycin D for 3 hours. The system y^+ activity increased by the arginine exposure was not blocked by the cycloheximide or actinomycin D in the incubation medium (Fig. 5-11).

5.3.10 System y^+ Activity Increased by Acute Arginine Exposure was Reversible

Caco-2 cells (2 days and 9 days old)were washed and incubated in the depletion medium ± 1 mM arginine for 3 hours. The cells which had been incubated with arginine were then washed three time with the depletion medium and incubated in the depletion medium ± 1 mM arginine for another 3 hours. System y^+ activity was increased following a 3 hours arginine incubation, and the increased was diminished after the cells were then incubated in depletion medium (lacking arginine) for 3 hours.

5.3.11 System y^+ Activity Increased by Arginine Exposure was a Kinetic Modification Effect

Caco-2 cells were washed three times with the depletion medium, and incubated in the same medium containing 1 mM arginine (1 mM mannitol as control) for 3 hours. The system y^+ uptake kinetics were measured over the [^{3}H]-arginine concentration ranging from 0.1 μM to 1 mM. The kinetics showed that both the V_{max} and K_m of the system y^+ activity was increased by the arginine pre-incubation (Fig. 5-13 & Fig. 4-14).

5.3.12 System y^+ Activity Increased by Arginine Chronic Exposure was not a Protein Synthesis-Dependent Process

The Caco-2 cells were washed with the depletion medium, and incubated in the same medium containing 0, 0.1, 1.0, or 10 mM arginine ± 10 μM cycloheximide or 6.6 μM chelerythrine for 24 hours. The medium was changed every 6 hours. The 5 μM arginine system y^+ activity was increased 7 fold by the arginine exposure. The degree of arginine uptake increased by the 0.1, 1.0 or 10 mM arginine incubation was the same. Cycloheximide or chelerythrine in the incubation medium did not block the system y^+ activity which was increased by the arginine incubation (Fig. 5-12).

In another study, Caco-2 cells pre-incubated with 1 mM arginine for 24 hours were then incubated in depletion medium lacking arginine for 3 hours. System y^+ activity increased by

the 24 arginine incubation returned to control level after the 3 hours starvation.

5.4 Discussion

system B and system y^+ activities were up-regulated by their own substrates, in contrast to substrate repression of transport activities found in other internal organs. System B and System y^+ were regulated independently.

5.4.1 Short-Term System B Activity Regulation by its Substrates

System B activity was up-regulated when cells were incubated with individual substrates. As shown in the Fig. 5-2, only the alanine, cystine, serine, threonine, and glutamine (that are transported by system B) induced the system B transport activity. Those non-system B substrates such as MeAIB, proline, lysine, arginine, and phenylalanine did not effect on System B activity. These data strongly suggest that the increase in System B activity following short-term amino acid exposure was a specific regulation. As will be discussed later in the following sections, System y^+ activity was up-regulated only by its own substrates. The fact that the System B substrates alanine, serine, cysteine, threonine did not inhibit nor induce System y^+ transport activity, and that, the System y^+ substrates lysine and arginine did not interfere System B transport nor induce the System B activity, indicated

that the System B and System y^+ activities were specifically and independently regulated only by their own substrates.

The fact that the System B activity could be induced by alanine within minutes and the increase was reversible, suggests that the increase in System B activity after the short-term substrate incubation could be due to: (1) a trans-stimulation of the transport systems, or/and (2) a trans-location of the transport units from cytosol to membrane. The insensitivity of cycloheximide or actinomycin D eliminated the possible involvement of de novo protein synthesis or new RNA synthesis mechanisms. Kinetic are valuable in identifying the trans-stimulation and the trans-location mechanisms. For a pure trans-location of transporter units, the only kinetic parameter that would change is V_{max} without K_m alteration. For the trans-stimulation mechanism, both the V_{max} and the K_m would be changed, and indeed in our kinetic studies, both the V_{max} and K_m were changed, favoring the notion that the acute activity increase was due to a trans-stimulation. We can not determine whether both the trans-stimulation and trans-stimulation were involved. In the future, by using the membrane vesicles pre-loaded with various concentrations of alanine, we will be able to determine if a trans-stimulation is involved. The only way to determine a role for tran-location is to probe transporter protein level using antibodies.

The System L activity increased by its own substrate

during short-term exposure, and this also likely involves a trans-stimulation phenomenon.

5.4.2 System B Activity Induced by Chronic Alanine Exposure

The cycloheximide- and chelerythrine-sensitive System B activity increased with the chronic alanine incubation. This differed from the short-term regulation, and suggested that de novo protein synthesis was involved, and that protein kinase C activation was involved. Because of the unavailability of specific probes such as antibodies or cDNA's at the time of this writing, it is not possible to determine whether the newly synthesized protein was the transporter protein, transporter regulatory protein, or some other regulatory protein. We predicted that the chronic activation effect would be a V_{max} effect without the modification of K_m or Hill number. In other words, the chronic alanine incubation stimulated the synthesis of System B transport-associated protein, resulting in a increase in functional transporter units. Alanine is a caloric amino acid and a carbon chain precursor for many metabolic intermediates, in addition to being non-toxic. The specific transporter activity stimulated by alanine was consistent with the observed in vivo up-regulation of intestinal epithelial amino acid transport (Diamond, 1991). The trans-stimulation provides an immediate safety margin at any given moment so that the cells can extract the maximum amount of nutrient from limited

availability. The specific up-regulation of transporter units could be an evolutionary adaptation which permitted animals to effectively adapt to their changing environment surroundings, provided that no toxic effects would occur.

5.4.3 System y^+ Activity Induced by its Own Substrate

System y^+ activity was induced after the cells were exposed to only the system y^+ substrates (Fig. 5-10). The inductive potency of these amino acids was directly related to the potency of these amino acids in inhibiting the system y^+ transport by analogue cross-inhibition. In other words, the amino acids which were transported by system y^+ induced the system y^+ activity (Figs. 4-6,7; Fig. 5-10). As we discussed in above, only the System y^+ substrate induced the System y^+ activity, and only System B substrates induced System B activity; there was no interference between the two systems. System B and System y^+ are specifically and independently regulated.

For the acute phase of System y^+ stimulation, the activity was not sensitive to cycloheximide. This rules out a mechanism of new transport protein synthesis. There are two acute activation mechanisms, trans-stimulation and trans-location. Our kinetic study unveiled changes in both the V_{max} and K_m for the substrate-induced System y^+, supporting the trans-stimulation theory. Future studies of acute stimulation should investigate phosphorylation event and measurement using

isolated membrane vesicles. Unlike System B, the System y^+ activity increased by chronic arginine exposure was not sensitive to cycloheximide or chelerythrine. The chronic arginine incubation did not induced a new transporter associated protein synthesis, and furthermore protein kinase C was not involved. This is consistent with the findings in other in vivo intestinal studies that showed that the intestinal essential lysine/arginine transport was not up-regulated in a long-term feeding related to the potential toxic effect of these essential amino acids. The transport capacity is determined by the factors such as the genetic hard-wiring or by the cell's needs other than the environmental availability. The trans-stimulation of the transporters provides the flexibility for maximum nutrient extraction at any given time within the safety margin.

5.5 Summary

The System B and System y^+ activities are up-regulated independently and specifically by only the substrates they transport. The system B is regulated in two phases: an acute trans-stimulation phase, and a chronic de novo protein synthesis- and protein kinase C-dependent phase. The System y^+ activity is only regulated by a substrate trans-stimulation. The difference between the System B and System y^+ substrate regulation may reflect the intrinsic properties of the transported substrates.

Fig. 5-1. System B activity in cells incubated in depletion medium with or without alanine

System B alanine (50 μM) uptake was measured in Caco-2 cells which had been incubated in depletion salt medium (± 1 mM alanine) for various period of time (0 - 6 hours). At incubation period ≥ 1 hour, the System B alanine uptake was significantly greater in cells incubated in salt plus 1 mM alanine than that incubated in salt only medium ($p < 0.05$, $n = 6$). Data shown were obtained in day 2 cells, with similar results obtained in day 9 cells.

Alanine uptake, nmole mg⁻¹ min⁻¹
1.5
1.0
0.5
0
60
120
180
240
300
360
Incubation time, minutes
Salt+1mM L-Ala
salt

Fig. 5-2. The acute effect of amino acids on System B activity

System B alanine (50 μM) uptake was measured in cells which had been incubated in DMEM salt medium, ± 1 mM amino acid for 3 hour. Amino acids alanine, serine, glutamine, cysteine, and threonine transported by System B induced the System B alanine uptake. Non-System B substrates such as phenylalanine, proline, arginine, and MeAIB did not induced the System B activity. 100% alanine uptake = 0.28 nmole/mg/min.

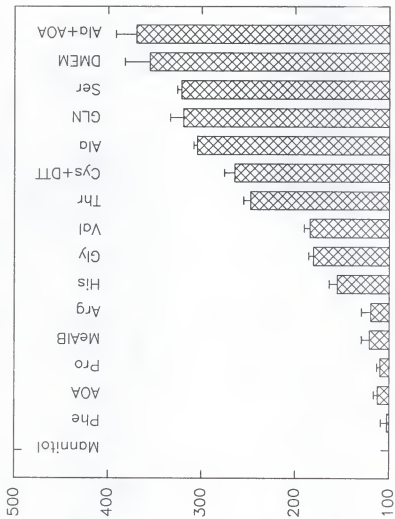

% of Alanine Transport
(Na^+ – dependent)
500
400
300
200
100
Mannitol
Phe
AOA
Pro
MeAIB
Arg
His
Gly
Val
Thr
Cys+DTT
Ala
GLN
Ser
DMEM
Ala+AOA

Fig. 5-3. The effect of AOA on System B activity

System B alanine (50 μM) uptake was measured in cells which had been incubated in salt medium, ± 1 mM alanine, 2.5 mM (aminooxy)acetic acid (AOA), or 1 mM alanine + 2.5 mM AOA for various periods of time (2 - 7 hours). Alanine alone and alanine plus AOA incubation stimulated the System B activity at each incubation period ($p < 0.05$, $n = 6$). AOA alone did not affect the System B activity after 2 and 4 hours incubation, but showed its stimulation effect after 7 hours incubation ($p < 0.05$, $n = 6$).

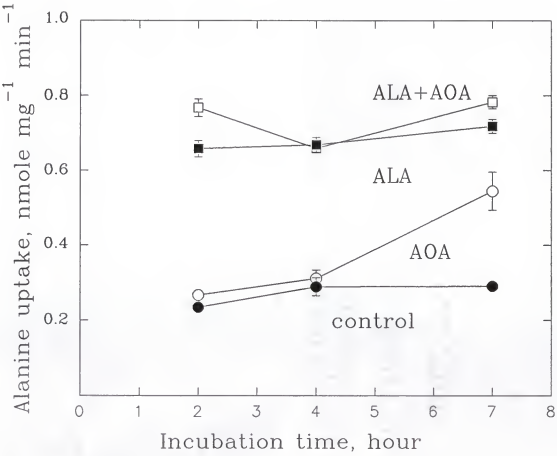

Alanine uptake, nmole mg^{-1} min^{-1}
1.0
0.8
0.6
0.4
0.2
0
1
2
3
4
5
6
7
8
Incubation time, hour
ALA+AOA
ALA
AOA
control

Fig. 5-4. The Effect of CHX on the acute alanine-stimulated System B activity

System B alanine (50 μM) uptake was measured in cells which had been incubated in salt medium (with or without 1 mM alanine), ± 50 μM cycloheximide in the incubation medium for 3 hours. The CHX incubation did not block the alanine induced System B alanine uptake ($p < 0.05$. $n = 9$).

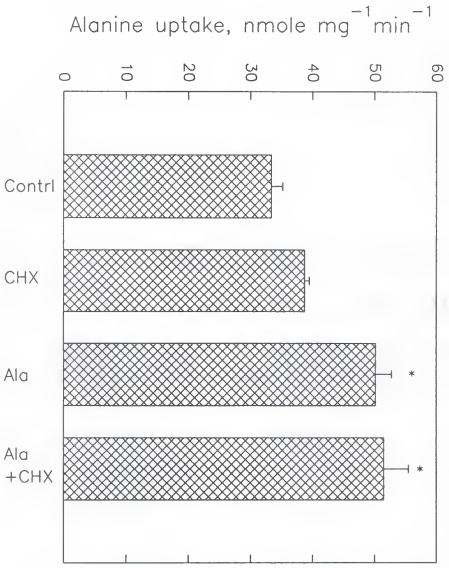

Alanine uptake, nmole mg^{-1} min^{-1}
0
10
20
30
40
50
60
Contrl
CHX
Ala
Ala
+CHX
*
*

Fig. 5-5. Kinetics of the acute alanine-stimulated System B activity

System alanine (10 μM - 5 mM) uptake was measured in cells (day 2) which had been incubated in salt medium, DMEM, 5 mM alanine in salt medium. In the salt incubation, V_{max} = 0.67 nmole/mg/min and K_m = 150 μmole alanine; in the DMEM and alanine incubation, V_{max} = 2.9 nmole/mg/min and K_m = 390 μmole alanine.

DMEM
5 mM ala
DMEM salt
J_{ala}, nmole mg^{-1} min^{-1}
J_{ala}/[alanine], μLiter mg^{-1} min^{-1}
0
1
2
3
4
5
6
7
8
0.5
1.0
1.5
2.0
2.5
3.0

Fig. 5-6. The acute effect of amino acids on MeAIB uptake

Na^+-dependent MeAIB (2.5×10^{-8} M) uptake was measured in cells (2 days old) after cells had been incubated in salt medium, or salt medium plus 1 mM MeAIB, 1 mM alanine, or 1 mM serine for 3 hours. The MeAIB incubation inhibited the MeAIB uptake ($p < 0.05$, $n = 3$), while alanine or serine incubation had not effect on the MeAIB uptake ($p > 0.05$, $n = 3$).

MeAIB uptake, 10^{-14} mole mg^{-1} min^{-1}
2.0
1.5
1.0
0.5
0.0
Control
MeAIB
Alanine
Serine

Fig. 5-7. The effect of chelerythrine on the chronic alanine-induced System B activity

System B alanine (50 μM) uptake was measured in cells (2 days old) which had been incubated in salt medium, salt medium plus various concentrations of alanine (0.1, 1.0, or 10 mM) with or without 6.6 μM chelerythrine in the incubation medium. The alanine incubation stimulated the System B alanine uptake ($p < 0.05$, $n = 6$), the stimulation was partially blocked by CHE ($p < 0.05$, $n = 6$). Similar results were obtained in 9 days old cells.

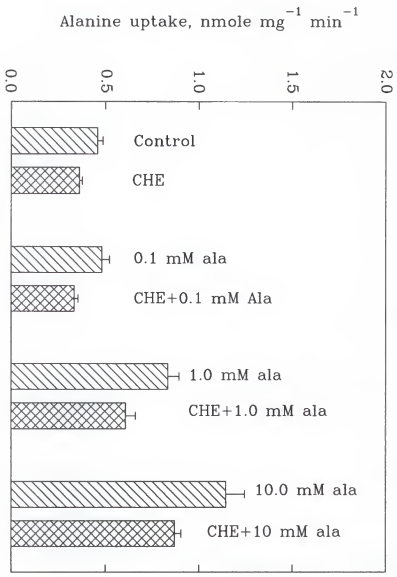

Alanine uptake, nmole mg^{-1} min^{-1}
0.0
0.5
1.0
1.5
2.0
Control
CHE
0.1 mM ala
CHE+0.1 mM Ala
1.0 mM ala
CHE+1.0 mM ala
10.0 mM ala
CHE+10 mM ala

Fig. 5-8. The effect of cycloheximide on the chronic alanine-induced System B activity

System B alanine (50 μM) uptake was measured in cells (2 days old) which had been incubated in salt medium (with or without 10 mM alanine), ± 10 μM CHX for 24 hours. The System B alanine uptake was stimulated by alanine incubation ($p < 0.05$, $n = 6$), and this stimulation was blocked by CHX ($p < 0.05$, $n = 6$). Similar results were obtained in 9 days old cells.

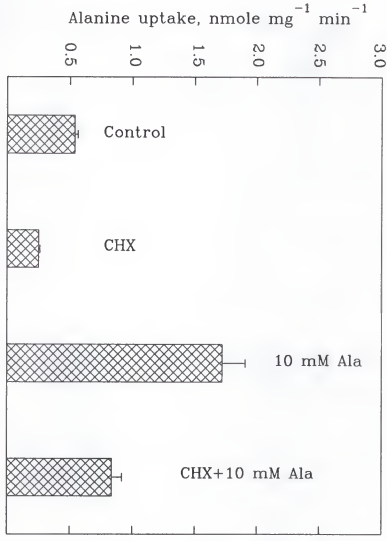

Alanine uptake, nmole mg^{-1} min^{-1}
0.5
1.0
1.5
2.0
2.5
3.0
Control
CHX
10 mM Ala
CHX+10 mM Ala

Fig. 5-9. System y^+ activity in cells incubated L-arginine or D-arginine

System y^+ arginine (5 μM) uptake was measured in cells (2 days old) which had been incubated in salt medium, 1 mM arginine in salt, or 1 mM D-arginine in salt for various periods of time (1 - 12 hours). The System y^+ arginine uptake was greatly stimulated by arginine incubation, D-arginine marginally increased the arginine uptake. Similar result were also obtained in 9 day s old cells.

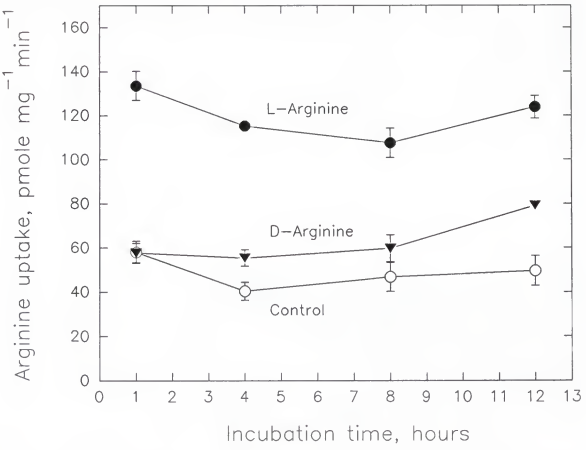

Arginine uptake, pmole mg^{-1} min^{-1}
160
140
120
100
80
60
40
20
0
L–Arginine
D–Arginine
Control
0 1 2 3 4 5 6 7 8 9 10 11 12 13
Incubation time, hours

Fig. 5-10. The acute effect of amino acids on System y^+ activity

System y^+ arginine (5 μM) uptake was measured in cells (2 days old) which had been incubated in salt medium, or salt medium plus 1 mM of various amino acids for 3 hours. The System y^+ arginine uptake was stimulated by 180% by system y^+ substrates lysine, ornithine, or arginine. Non-system y^+ substrates alanine, serine etc did not stimulated the arginine uptake. Similar results were also obtained in 9 days old cells.

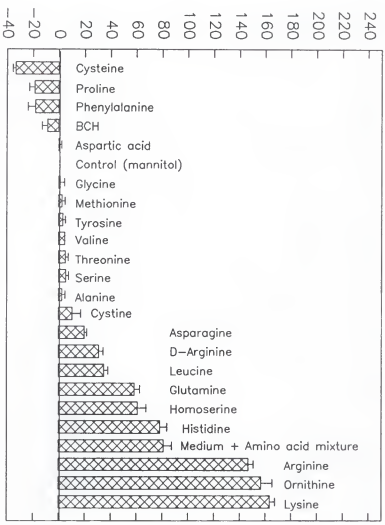

% Stimulation of Arginine Uptake
-40
-20
0
20
40
60
80
100
120
140
160
180
200
220
240
Cysteine
Proline
Phenylalanine
BCH
Aspartic acid
Control (mannitol)
Glycine
Methionine
Tyrosine
Valine
Threonine
Serine
Alanine
Cystine
Asparagine
D-Arginine
Leucine
Glutamine
Homoserine
Histidine
Medium + Amino acid mixture
Arginine
Ornithine
Lysine

Fig 5-11. The effect of CHX on the acute arginine-stimulated System y^+ activity

System y^+ arginine (5 μM) uptake was measured after cells (2 days old) had been incubated in salt, 1 mM arginine, 50 μM CHX, or 1 mM arginine plus 50 μM CHX for 3 hours. The arginine uptake was stimulated by the arginine incubation ($p < 0.05$, $n = 6$), CHX had no effect on the induction ($p . 0.05$, $n = 6$). Similar results were observed in 9 day old cells.

Arginine uptake, pmole mg^{-1} min^{-1}
200
160
120
80
40
0
Control
CHX
Arg
*
Arg+
CHX
*

Fig. 5-12. The effect of CHX and chelerythrine on the chronic arginine-induced system y^+ activity

System y^+ arginine (5 μM) uptake was measured after cells (2 days old) had been incubated in salt medium, salt medium plus various concentrations of arginine (1, or 10 mM), ± 10 μM CHX or 6.6 μM CHE for 24 hours. The arginine uptake was increased by arginine incubation, CHX or CHE did not block this arginine induction.

Arginine uptake, pmole mg^{-1} min^{-1}
400
350
300
250
200
150
100
50
0
[Arg]
0 mM
1 mM
10 mM
Control
0 mM
1 mM
10 mM
+ CHX
0 mM
1 mM
10 mM
+ CHE

Fig. 5-13. Kinetics of acute arginine-stimulated System y^+ activity in salt medium

System y^+ arginine (0.1 μM - 1 mM) uptake kinetics were measured after cells (2 days old) had been incubated in salt medium or 1 mM arginine in this medium for 3 hours. For the cells incubated with salt only, the V_{max} = 0.25 nmole/mg/min and K_m = 31 μmole arginine; for the arginine incubation group, V_{max} = 2.75 nmole/mg/min and K_m = 81 μmole arginine.

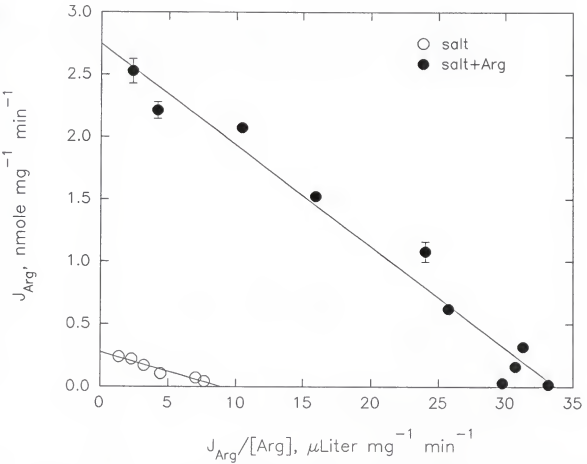
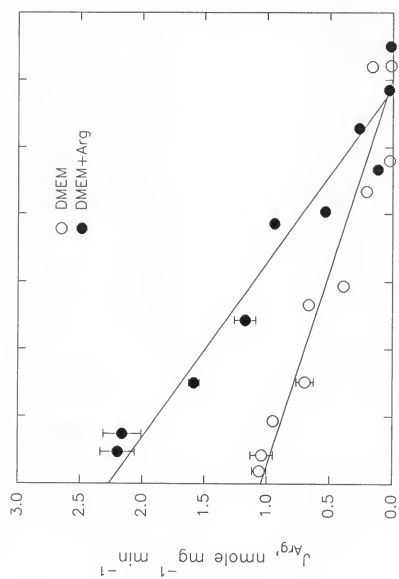

3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
25
30
35
salt
salt+Arg
J_{Arg}, nmole mg^{-1} min^{-1}
$J_{Arg}/[Arg]$, μLiter mg^{-1} min^{-1}

Fig. 5-14. Kinetics of acute arginine- or DMEM-stimulated System y^+ activity

System y^+ arginine (0.1 μM - 1 mM) uptake kinetics were measured after cells had been incubated in DMEM or DMEM plus 1 mM arginine for 3 hours. For the DMEM incubation, V_{max} = 1.05 nmole/mg/min and K_m = 39 μmole arginine; for the arginine/DMEM incubation, V_{max} = 22.7 nmole/mg/min and K_m = 79 μmole arginine.


DMEM
DMEM+Arg
J_{Arg}, nmole mg^{-1} min^{-1}
J_{Arg}/[Arg], μLiter mg^{-1} min^{-1}
0.0
0.5
1.0
1.5
2.0
2.5
3.0
0
5
10
15
20
25
30
35

CHAPTER 6
THE EFFECTS OF PEPTIDE GROWTH FACTORS ON SYSTEM B AND SYSTEM y+ TRANSPORT ACTIVITIES

6.1 Introduction

Epidermal growth factor (EGF), is a member of the growth factor family, which has been intensively studied over the past 30 years (Hernandez-Sotomayor & Carpenter, 1992). The protein structure, gene expression, biological function, and the molecular regulation of EGF and the EGF receptor are well understood. Transforming growth factor-alpha (TGFα) is structurally similar to EGF (Montelione et al., 1988, 1989; Caver et al., 1986; Mayo et al., 1989). The structure of EGF and TGFα is reported to be related to their functions (Capenter & Wahl, 1990).

EGF/TGFα command their function through a binding to the EGF membrane receptor. The EGF receptor is a glycoprotein composed of three major domains: an extracellular hormone binding domain, a hydrophobic transmembrane region, and a cytoplasmic domain. TGFα also binds to the same EGF receptor.

EGF receptor belongs to the tyrosine kinase family. The EGF/TGFα binding to the EGF receptor induces a rapid reversible changes in the receptor tyrosine kinase activity causing an auto-phosphorylation of the EGF receptor and the

phosphorylation of other receptor's substrates. The tyrosine kinase activity is essential for the EGF receptor biological activity (Chen et al., 1987; Glenney et al., 1988; Honnerger et al., 1987; Moolenaar et al., 1988)). The EGF receptor substrates include: PLC-γ1 (Margolis et al., 1989; Meisenhelder et al., 1989; Wahl et al., 1989), GAP (Ellis et al., 1990; Molloy et al., 1990), lipocortin I (Fava & Cohen, 1984), c-erbB-2 (Akiyama et al., 1988; Stern & Kemps, 1988), and PI-3 kinase (Whittman et al., 1988).

One action of the activated EGF receptor kinase is to phosphorylate the phospholipase C (PLC) which hydrolyzes the phosphatidylinositol-4,5-biphosphate to produce inositol-1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DAG). IP_3 and DAG stimulate the increases in the intracellular free Ca^{2+} and protein kinase C activity, respectively. Protein kinase C then causes various biological responses, mainly gene-expressed related mechanisms through a series unknown pathways.

The EGF biological responses are of two types. The first concerns a rapid signal transduction via the receptor tyrosine auto-phosphorylation, acting in concert with the calcium released from the intracellular stores, and leading to the phospholinositide hydrolysis, with subsequent formation of diacylglycerol (Carpenter et al., 1979; Moolenaar et al., 1984; Morris et al., 1984; Johnson et al., 1986; Wahl & Carpenter, 1988a; Smith et al., 1983; Serreo, 1987). The second mechanism involves long term biological responses such

as stimulation of cell proliferation, inhibition of differentiation, stimulation of oocyte maturation, stimulation of vasoconstriction. (Raymond et al., 1986; Centrlla, 1987; Reilly et al., 1987; Kim et al., 1987; Downs et al., 1988; Berk et al., 1985; Muramatsu et al., 1985, 1986). Depending on the cell type and the physiological circumstances, EGF exerts many biological functions (Carpenter & Wahl, 1990). One of the most dramatic biological effects of EGF is the regulation of cell growth and differentiation, especially in epithelial cells and tissues (Carpenter & Wahl, 1990).

The EGF receptors were reportedly found at developing intestinal epithelial cells and the human Caco-2 cell line. In the Caco-2 cell line there is a greater density of receptors in less differentiating cells. Two-thirds of the EGF receptors appear on the basolateral membrane, while the one-third on the apical membrane. The K_d of the EGF receptor in the Caco-2 cell is 0.6 μM EGF (Hidalgo et al., 1989; Koyama & Podolsky, 1989).

EGF is normally present in the intestinal mucosa and in the lumen. The major sources are from the submaxillary gland secretion, from the Brunner's glands of the duodenum, from the jejunal/ileal mucosa, and from exogenous sources such as milk (which contains 40 -400 ng/ml EGF). EGF and TGFα are extremely stable in the presence of the gastric acid and the intestinal digestive enzymes (Britton et al., 1989; Potter, 1990). The secretary sources for the EGF in blood stream are still unknown.

Insulin, glucagon, adenosine induce System A transport activity via a transcription and translation mechanism in hepatocyte (La Cam & Freychet, 1978; Cariappa & Kilberg, 1990; Kiyokawa et al., 1991).

Despite the understanding of many EGF biological activities, the effect of EGF on the intestinal absorption of amino acids has not been explored.

6.2 Methods And Materials

6.2.1 Methods

6.2.1.1 Treatments with EGF/TGFα, cycloheximide, chelerythrine, and H-7/calphostin C

The protocols to treat Caco-2 cells were basically the same for each of the various agents. Serum-free DMEM was prepared by supplementing Sigma's DMEM with non-essential amino acids, penicillin and streptomycin, but not serum. Treatments began with a 2 hour preincubation of cells in the depletion medium. The cells were then exposed to various buffers for various times described below. Every 6 hours, buffers were removed and replenished with the same buffers to ensure a constant agent concentration and to eliminate a possible autocrines released into the medium from the cells. Caco-2 cells remained healthy during at least 48 hours exposure to our depletion medium.

Experiments with EGF/TGFα. The cell treatments were: (i) control group, serum-free DMEM plus the same amount of 0.1 M

acetic acid vesicle as used in the EGF/TGF∝ treatment (< 0.5% of the medium volume), (ii) treatment with EGF/TGF∝, DMEM plus various concentration of (EGF/TGF∝ is diluted from stocks in 0.1 M acetic acid stored at 4°C), and (iii) treatment with EGF/TGF∝ with additional agents, DMEM plus EGF/TGF∝ plus agent specified in the text, with each specified agent in DMEM as control. Cell were exposed to the treatments up to 48 hours.

Cycloheximide (CHX) treatments. The cells were treated with: (i) control group, serum-free medium, (2) treatment with CHX, DMEM plus 10 - 100 μM CHX (CHX was prepared in aqueous solutions made the day of the experiment), (iii) treatment with CHX and other agents, DMEM plus 10 - 100 μM CHX with specified agent added in to the medium, internal control used DMEM plus specified agent. The treatments were for various lengths of time.

Chelerythrine Cl experiments. The cells were treated with: (i) control group, only serum-free DMEM, (ii) treatment with chelerythrine, serum-free DMEM plus chelerythrine (chelerythrine Cl was diluted from stocks in H_2O stored at -20°C), (iii) treatment with chelerythrine Cl and specified agents, DMEM plus chelerythrine plus specified agent, with DMEM plus specified agent as internal control.

Calphostin C treatments. Cells were treated with: (i) control group serum-free DMEM plus the same amount of DMSO as appeared in the calphostin C solution (<1% of the medium volume), (ii) treatment with calphostin C, serum-free DMEM

plus various concentrations of calphostin C (calphostin C was diluted from stock DMSO solution stored at -20°C), (iii) treatment with calphostin C and other agent, serum-free DMEM plus calphostin C plus specified agent, with DMEM plus specified agent as internal control. These groups were continuously exposed to a 20 watt fluorescent light in the 37°C humidified incubator to photo-activate the calphostin C.

H-7 treatments. The cells were treated with: (i) control group, serum-free medium plus the same amount of DMSO as appeared in H-7 (<1% of the medium volume), (ii) treatment with H-7, serum-free DMEM plus H-7 (H-7 was diluted from a DMSO stock solution stored at -20°C), (iii) treatment with H-7 and other agents, serum-free DMEM plus H-7 plus the specified agent, with DMEM plus the specified agent as internal control.

6.2.2 Materials

TGF∝ (human recombinant) and EGF (human recombinant) were obtained from Promega Co., Madison, WI. Chelerythrine Cl and Calphostin C were from LC services Co., Woburn, MA. H-7, cycloheximide, medium and other chemicals were from Sigma Co., St. Louis, MO.

6.3 Results

6.3.1 The Effect of EGF/TGFα on System B Activity

The Caco-2 cells were pre-incubated with 20 ng/ml or 100 ng/ml EGF in the serum-free medium for various length of time (0 - 48 hours). The medium was changed every 6 hours. The System B activities were measured at 5 minutes, 10 minutes, 30 minutes, 1, 2, 4, 8, 12, 18, 24, 30, 41, and 48. System B activities were not affected by TGFα/EGF at the incubation time less than 30. After 48 hours of continuous incubation, TGFα increased the System B activity by 75%, while EGF stimulated at least 57% (Fig. 6-1). Cycloheximide (10 μM) or 6.6 μM chelerythrine in the incubations medium blocked the System B activity increased by TGFα/EGF (Fig. 6-1).

In separate experiments, the Caco-2 cells were pre-treated with insulin, glucagon, dexamethasone, or TGFβ for 0 - 48 hours. The System B activity was not affected by either insulin, glucagon, or dexamethasone. In contrast to TGFα, TGFβ (>18 hours continuous incubation) inhibited the System B activity.

In separate experiments, other protein kinase C inhibitors H-7 and calphostin C were added to the TGFα/EGF incubation media. Calphostin C (50 nM) blocked the TGFα/EGF System y^+ stimulation effect, while the H-7 (200 μM) did not have any effect.

6.3.2 The EGF/TGF∝ Pulse Stimulation Effects

The Caco-2 cells were pre-incubated with TGF∝ (20 ng/ml) or EGF (100 ng/ml) for 2 hours, and incubated in the serum-free medium (lacking TGF∝/EGF) for the remaining 46 hours. The System B activity was not increased by the TGF∝/EGF pulse treatments.

6.3.3 The TGF∝/EGF Effect on the System y^+ Activity

The Caco-2 cells were pre-treated with TGF∝ (20 ng/ml) or EGF (100 ng/ml) in serum-free medium for various length of time (0 - 48 hours). Like the System B, the System y^+ activity was increased by TGF∝/EGF only after 30 hours of continuous incubation. At 48 hours of incubation, TGF∝ increased the System y^+ arginine (5 μM) uptake by 80% and EGF increased the arginine uptake by 70% (Fig. 6-2). The addition of 10μM cycloheximide or 6.6 μM chelerythrine in the TGF∝ or EGF incubation medium blocked the TGF∝/EGF stimulation effects (Figs. 6-3, 6-4). The addition of 50 nM calphostin C also blocked the TGF∝/EGF effects. H-7 (later disclaimed as a defective H-7 isomer by Sigma) did not have any effect on the System y^+ activity.

6.3.4 The TGF∝/EGF Pulse Effect on System y^+

The Caco-2 cells were pre-incubated with TGF∝ (20 ng/ml) or EGF (100 ng/ml) for 2 hours, and then incubated in the same serum-free medium (lacking TGF∝/EGF) for the remaining 46

hours. System y^+ arginine uptake was unaffected by this treatment.

6.4 Discussion

The peptide growth factors TGF∝ and EGF both stimulated the System B and System y^+ activities in the Caco-2 cells at both the undifferentiated and differentiated cells.

In addition to being a the slow process requiring many hours (>30 hours), the TGF∝/EGF effect was cycloheximide-sensitive. These combined data suggest that it is unlikely that the TGF∝/EGF effect was caused by rapid phosphorylation of transporter protein. A de novo protein synthesis process was likely involved in the TGF∝/EGF stimulation of System B and System y^+. But whether the synthesized protein was the transporter protein, regulatory protein, or other protein was unknown. Future studies using a System y^+ antibody probe may provide a more precise answer. Normally, de novo protein synthesis can occurs within several hours, and it is not clear why TGF∝/EGF took more than 30 hours to show their effect. A cascade of regulatory processes may be involved, in addition to protein synthesis.

The protein content and cell numbers of the 48 hour CHX-treated cells was comparable to the pre-CHX-treatment level. The viability of CHX-treated cells was >99%. Compared to the control group (only DMEM treatment), the CHX-treated cells had 40% less protein and 40% less cells. So the CHX's inhibitory

effect on the System B and System y^+ activities was likely inhibiting new protein synthesis rather than cytotoxic effect.

Prolonged and continuous exposure to TGF∝/EGF was required for the System B and System y^+ activity stimulation. The mechanism for the delay is unknown. In the light of a System A regulatory mechanism proposed by Engleberg (1986), the System B and System y^+ could be regulated by at least two groups of regulatory forces which are always present and are in equilibrium. TGF∝/EGF shift the balance to the stimulatory side, resulting in synthesis of transporter protein or other regulatory proteins, while the negative regulatory force always tries to bring the balance back.

As we discussed above in the introduction section, TGF∝/EGF bind to the EGF receptor and the TGF∝/EGF-receptor complex phosphorylates many substrates of the EGF receptor. The substrates may include the receptor itself, the ras GTPase-activating protein or GAP, PI-3 kinase, and PLCγ1. Which signal pathway did TGF∝/EGF participate in activating Systems B and y^+ activities? The inhibition of the TGF∝/EGF stimulation effect on the alanine and arginine transport by the specific protein kinase C inhibitors chelerythrine and calphostin C suggested that the protein kinase C activation was involved in the process. Chelerythrine CL specifically inhibits PKC by acting at PKC's catalytic subunits, while calphostin C binds at PKC's regulatory subunits (Tamaoki et al., 1990). We have found that the H-7 isomer defectively

manufactured by Sigma Co as H-7 did not influence the TGF∝/EGF effect on System B and System y^+ activities. The phospholipase Cγ (PLC) was likely a major intermediate phosphorylated by TGF∝/EGF in the System B and System y^+ transport activation. PLC hydrolysizes phosphatidylinositol-4,5-biphosphate (PIP_2), generating the intracellular second messenger diacylglycerol (DAG) which is an endogenous activator of protein kinase C. Further study of the PIP_2 level, diacylglycerol level, and the inositol hydrolysis in cells will provide the information concerning details of the pathways. It is still unclear if the PLC pathway is the only pathway for this stimulation.

Because the protein kinase C inhibitors also inhibited general protein synthesis, it is likely that the protein kinase C activation precedes the de novo protein synthesis associated with the System B and System y^+ activation.

It is notable that the Na^+-independent alanine System L transport was not stimulated by the TGF∝/EGF incubation. In fact, due to the stimulation effect of TGF∝/EGF on cell protein synthesis, the System L activity per mg protein actually decreased. These findings support the notion that System B and System y^+ activation is a selective event.

Unlike the transport substrate regulation we discussed in the preceding chapter, TGF∝/EGF stimulated both the System B and System y^+ transport activities. This phenomenon may be associated with the stimulation effect of TGF∝/EGF on the epithelial proliferation, cell growth, or mitogenesis.

TGF∝/EGF increased the needs of cells for amino acids for their growth.

6.5 Summary

Prolonged, continuous exposure to peptide growth factors TGF∝ or EGF stimulates System B and System y^+ activities in Caco-2 cell in both the undifferentiated and differentiated states. The stimulatory effect involves a de novo protein synthesis process. Whether this involves the transporter protein or a regulatory protein is not clear. The intracellular protein kinase C activation is involved in the pathway of TGF∝/EGF activation of the System B and System y^+ transport activities, thereby suggesting the possibility of a role for PLC-γ phosphorylation.

Fig. 6-1. The effects of chronic TGF∝, or EGF System B activity

System B alanine (50 μM) uptake was measured in cells (2 days old and 9 days old), after cells had been incubated in DMEM, 20 ng/ml TGF∝, or 100 ng/ml EGF for 48 hours. TGF∝ or EGF each stimulated the alanine uptake ($p < 0.05$, $n = 12$).

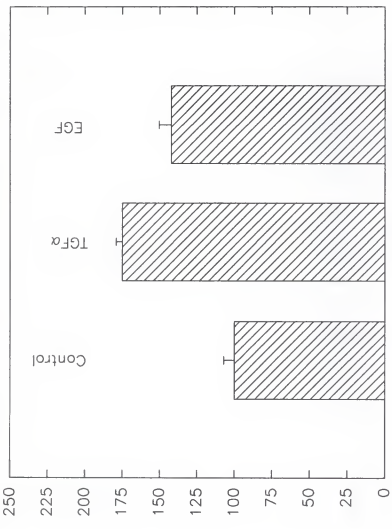

Alanine uptake, per cent
250
225
200
175
150
125
100
75
50
25
0
Control
TGFα
EGF

Fig. 6-2. The effect of CHE on the chronic TGF∝/EGF-induced System B activity

System B alanine (50 μM) uptake was measured after cells had been incubated in DMEM, TGF∝ or EGF, ± 6.6 μM CHE for 48 hours. TGF∝ or EGF each stimulated the alanine uptake, CHE in the TGF∝ or EGF medium blocked this stimulative effect ($p < 0.05$, $n = 6$)

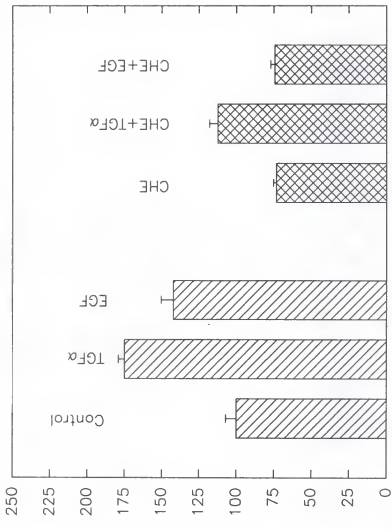

Alanine uptake, per cent
250
225
200
175
150
125
100
75
50
25
0
Control
TGFα
EGF
CHE
CHE+TGFα
CHE+EGF

Fig. 6-3. The effectof CHX on the chronic TGF∝/EGF-induced System B activity

System B alanine uptake was stimulated by 48 hours of continuous TGF∝ or EGF incubation. CHX (10 μM) in the TGF∝ or EGF medium blocked the stimulation effects ($p < 0.05$, $n = 12$). Data shown were from day 2 cells, with similar results obtained in day 9 cells. 100% alanine uptake = 0.8 nmole/mg/min.

Alanine uptake, per cent
250
225
200
175
150
125
100
75
50
25
0
Control
TGFα
EGF
CHX
CHX+TGFα
CHX+EGF

Fig. 6-4. The chronic effect of TGFα or EGF on System y^+ activity

System y^+ arginine (5 μM) uptake was measured in day 2 cells which had been incubated in DMEM, TGFα, or EGF in DMEM for 48 hours. TGFα and EGF each stimulated the System y^+ arginine uptake ($p < 0.05$, $n = 6$). Similar results were obtained in 9 days old cells.

Arginine uptake, pmole mg^{-1} min^{-1}
300
250
200
150
100
50
0
Control
EGF
TGF$_\alpha$

Fig. 6-5. The effects of chelerythrine on the chronic TGF∝/EGF-induced System y^+ activity

System y^+ arginine (5 μM) uptake was measured after cells (2 days old) had been incubated in DMEM, TGF∝, or EGF, ± 6.6 μM CHE for 48 hours. TGF∝ and EGF each stimulated the arginine uptake, CHE in the TGF∝/EGF incubation media blocked this stimulation effect ($p < 0.05$, $n = 6$).

Arginine uptake, pmole mg⁻¹ min⁻¹
300
250
200
150
100
50
0
Control
CHE
EGF
EGF + CHE
TGFα
TGFα + CHE

Fig. 6-6. The effect of CHX on the chronic TGF∝/EGF-induced System y^+ activity

System y^+ arginine (5 μM) uptake in cells (2 days old) was stimulated by 48 hours TGF∝ or EGF incubation. CHX (10 μM) in the TGF∝/EGF incubation blocked this TGF∝/EGF stimulation effect ($p < 0.05$, $n = 6$).

Arginine uptake, pmole mg^{-1} min^{-1}
0
50
100
150
200
250
300
Control
CHX
EGF
EGF + CHX
TGFα
TGFα + CHX

CHAPTER 7
THE EFFECTS OF PHORBOL ESTERS ON SYSTEM B AND SYSTEM y^+ TRANSPORT ACTIVITIES

7.1 Introduction

The tumor promotors phorbol 12-myristate 13-acetate (TPA), or phorbol 12,13-dibutyrate (PDBU), have long been known for their effects in promoting mitogenesis and cell growth. The phorbol ester effect is through a series of an intracellular cascade processes that is initiated by protein kinase C activation. The lipophilic phorbol esters diffuse through the cell membrane as a substitute for DAG in directly activating the intracellular protein kinase C. Even though there have been reports that phorbol esters are not specific protein kinase C activators, and that they may activate other processes, phorbol esters are widely used to activate protein kinase C.

Protein kinase C has two subunits: a regulatory subunit, and a catalytic subunit. Diacylglycerol and phorbol esters, bind to the regulatory subunit causing a structure change which activates or inactivates the kinase. Once the protein kinase C is activated, the catalytic subunit bind to its substrate and catalyzes it. Both steps are essential for the biological functions.

Many protein kinase C inhibitors have been developed, and are classified according to their working mechanisms. Inhibitors such as H-7 and calphostin C bind to the regulatory side, while inhibitors such as chelerythrine bind to the catalytic subunit. Either of above step will block the PKC activation.

Calphostin C blocks the binding of phorbol ester to the PKC when photo-activated by a fluorescent light. Calphostin C is exceptionally selective for PKC, with inhibitory concentration much less than that required to inhibit protein kinase A or other protein tyrosine kinases (Kobayashi et al., 1989; Bruns et al., 1991).

Chelerythrine inhibits protein kinase C activity at a concentration over 100 times lower than that for inhibition of protein kinase A, protein tyrosine kinases, or the Ca^{++}-calmodulin-dependent protein kinases (Herbert et al., 1990).

Therefore, both the calphostin C and chelerythrine are excellent protein kinase C inhibitor with high selectivity.

7.2 Methods And Materials

7.2.1 Pre-treatment with Phorbol Esters

The Caco-2 cells were washed three time with the serum-free-medium, and incubated in the dark or under fluorescent light with the same medium containing: (1) control group, serum-free DMEM plus the same amount of DMSO as appeared in the phorbol esters (DMSO was < 0.5% of the medium volume), (2)

treatment with phorbol esters, serum-free DMEM plus various concentration of TPA or PDBU (1 pM - 10 μM), (TPA and PDBU were diluted from DMSO stock solution and stored at -20°C), (3) treatment with phorbol esters and other agents, DMEM plus phorbol ester plus additional other chemicals such as cycloheximide, chelerythrine, calphostin C, with DMEM plus other agent as an internal control, chelerythrine and calphostin C were from LC Services Co., Woburn, MA.). The cells were incubated for various periods of time in the 37°C incubator. The medium was changed very 6 hours. For the calphostin C incubation, a 20 watt fluorescent light was placed in the incubator. The details of each incubation are explained below.

7.2.2 Pre-treatment with Dibutyryl Cyclic-AMP (dcAMP)

The Caco-2 cells were washed three times with the serum-free medium, and incubated with the same medium with ± 0.5 μM dcAMP for various times (0 - 24 hours). The medium was changed every 6 hours.

7.3 Results

7.3.1 The Phorbol Ester (TPA) Stimulation of the System B Activity Time Course

The Caco-2 cells were pre-incubated with 0.5 μM TPA in the serum-free medium for various length of time (0 - 24 hours) prior to the uptake experiments. The medium was changed

every 6 hours. The 50 μM alanine system B uptake activity was measured immediately after each incubation point. The system B alanine uptake activity was stimulated by a prolonged TPA incubation (≥ 8 hours). The stimulation effect increased steadily up to at least 24 hours (Fig. 7-1). At incubation times less than 8 hours, there was no TPA effect on the system B activity.

The system L alanine uptake was not stimulated by TPA incubation (0 - 24 hours). Due to the increase cell protein synthesis, the system L alanine uptake per mg protein actually decreased.

7.3.2 TPA Pulse Effect on System B Activity

The Caco-2 cells were pre-treated with 0.5 μM TPA for 0 - 2 hours, and then were washed and incubated in the serum-free medium (lacking TPA) for the remaining time period (22 hours). The total incubation time was 24 hours, including the TPA incubation plus the following non-TPA incubation. System B activity was then measured immediately after the total incubation. Unlike their continuous incubation counterparts, the pulse incubation did not affect the system B activity.

7.3.3 Dose Response for Phorbol Ester (TPA) Stimulation of System B

The Caco-2 cells were pre-incubated in the serum-free medium with various concentration of TPA (1 pM - 10 μM) for 24 hours prior to the uptake experiments. The 50 μM alanine

System B uptake activity was then measured immediately after each incubation. System B alanine uptake activity was stimulated at concentrations of [TPA] ≥ 10 nM. A peak stimulation of 2 fold was observed at [TPA] = 1μM, and the stimulation effect was attenuated at [TPA] = 10 μM (Fig. 7-2).

7.3.4 Phorbol Ester Stimulated the System B Activity Regardless the Cell Age

The Caco-2 cells (1 day old through 35 days old) were pre-incubated with 0.5 μM TPA in the serum-free medium for 24 hours prior to the uptake experiments. The system B activity was stimulated at least 2 fold by TPA at all cell ages (Fig. 7-3). At each cell ages, System L uptake was not significantly altered by TPA.

7.3.5 The TPA Stimulation of System B Activity Involved De Novo Protein Synthesis

Caco-2 cells were pre-incubated in serum-free medium containing 0.5 μM TPA, ± 10 μM CHX for 24 hours prior to the uptake experiments. The 50 μM alanine System B uptake activity was stimulated 2 fold by the TPA incubation. The addition of CHX in the TPA incubation medium blocked the stimulation effect. CHX alone did not significantly affect System B activity (Fig. 7-4).

Caco-2 cells were also pre-incubated with 0.5 μM TPA in serum-free medium, ± 0.5 μM actinomycin D for 24 hours. System B activity increased following TPA exposure. The incubation in

TPA/actinomycin D had no affect on System B activity (Fig. 7-5).

7.3.6 Phorbol Ester Stimulation of System B Activity was via PKC Activation

The Caco-2 cells were pre-incubated with 0.5 μM TPA in the serum-free medium, ± 6.6 μM chelerythrine or 50 nM calphostin C in the medium for 24 hours prior to the uptake experiments. The 50 μM alanine System B uptake activity was measured. The system B activity increased 2 fold following the TPA incubation alone. The TPA/chelerythrine or the TPA/calphostin C incubation did not affect System B activity (Fig. 7-6). H-7 isomer did not alter the System B activity nor the effect of TPA.

7.3.7 The Effect of Phorbol Ester on the System B Transport Kinetics

The Caco-2 cells were pre-incubated with 0.5 μM TPA in the serum-free medium for 24 hours prior to the uptake experiments. The system B alanine transport kinetics ([^{3}H]-alanine = 1 μM - 5 mM) were then measured (Figs. 7-7). The V_{max} of day 2 cells was significantly increased 2 fold by TPA (V_{max} = 3.05 nmole/mg/min in the control cells, V_{max} = 5.9 nmole/mg/min with the TPA treatment). The V_{max} of day 9 cells was increased 3 fold by TPA incubation (V_{max} = 0.5 nmole/mg/min in the control cells, Vmax = 1.65 nmole/mg/min with the TPA treatment). The Km (K_m = 160 μM alanine) was the same

regardless the age and the TPA treatment (Fig. 7-8).

7.3.8 Phorbol Ester Up-Regulated the System y^+ Uptake Activity

The Caco-2 cells were pre-incubated with 0.5 μM TPA in serum-free medium for various length of time (0 - 24 hours) prior to the uptake experiments. The System y^+ activity was stimulated by TPA only after a prolonged incubation; at least 8 hours were required for the effect. The TPA stimulation effect increased steadily up to at least 24 hours. A 24 hours TPA incubation period was chosen for the subsequent TPA experiments.

7.3.9 The TPA Pulse Effect on System y^+ Activity

The Caco-2 cells were pre-incubated with 0.5 μM TPA in the serum-free medium for various periods of time (0 - 2 hours), then re-incubated in the serum-free medium (lacking TPA) for the remaining periods prior to the uptake measurements. The total incubation time was 24 hours, including the pulse TPA incubation plus the following non-TPA incubations. The TPA pulse treatments alone did not affect the system y^+ activity.

7.3.10 TPA's Effect on the System y^+ Activity at Various Cell Ages

The Caco-2 cells (1 day old - 14 day old) were pre-treated with 0.5 μM TPA in the serum-free medium for 24 hours prior to the arginine uptake experiments. The 5 μM arginine

uptake activity was stimulated up to 2 fold by TPA at all cell ages (Fig. 7-9).

7.3.11 The TPA Stimulation of System y^+ Activity Involved De Novo Protein Synthesis

The Caco-2 cells were pre-incubated in serum-free medium with 0.5 μM TPA for 24 hours, ± 50 μM or 20 μM cycloheximide including in the incubation medium for various windows of time (first 6 hours, second 6 hours, third 6 hours, fourth 6 hours, first 12 hours, first 18 hours, and the entire 24 hour period). Fig. 7-10 shows that the 5 μM arginine system y^+ uptake activity was stimulated 50% by TPA incubation, but the stimulation was not retarded by each of the 6 hour CHX incubation. However, the first 12 and 18 hour periods of CHX/TPA incubation blocked the TPA stimulation of System y^+ activity. The absolute uptake activity was decreased following 24 hours of CHX incubation, but due to the greater decrease in cell protein, the activity per mg protein was not affected.

7.3.13 The Phorbol Ester Stimulation of System y^+ Activity was Inhibited by Specific Inhibitor of Protein Kinase C

The Caco-2 cells were pre-incubated with TPA in the serum-free medium, ± 6.6 μM chelerythrine or 50 nM calphostin C for 24 hours prior to the uptake measurements. System y^+ activity was increased by TPA alone. The TPA/chelerythrine or TPA/calphostin C combination incubation did not stimulate System y^+ activity, and chelerythrine or calphostin C alone

did not affect activity (Fig. 7-11).

7.3.14 The Effect of Phorbol Ester on the System y^+ Transport Kinetics

The Caco-2 cells were pre-treated with TPA in the serum-free medium for 24 hours prior to the uptake experiments, and the system y^+ arginine transport kinetics were measured ([^{3}H]-arginine = 0.1 μM - 1 mM). The V_{max} was increased by TPA in both the day 2 and day 9 cells (V_{max} = 777 pmole/min/min in day 2 cell with V_{max} = 1111 pmole/mg/min in TPA treated day 2 cells, V_{max} = 541 pmole/mg/min in day 9 cells and V_{max} = 720 pmole/mg/min with TPA treatment). The K_m of 43.3 and 55 μM arginine was the same regardless the cell age and the TPA incubation (Fig. 7-12).

7.4 Discussion

The phorbol ester TPA stimulated both System B and System y^+ activities. The TPA effects on transporter activities were similar in terms of the potency, the onset of stimulation, and the dependency of de novo protein synthesis.

TPA diffuses through cell membrane and directly binds to the protein kinase C regulatory domain, the endogenous diacylglycerol binding site. TPA possesses structural similarities with diacylglycerol, and both activate protein kinase C. TPA can acutely activate protein kinase C, or chronically inactivate PKC by depleting the cell of active membrane-bound form of PKC.

In the last chapter, we discussed that TGF∝/EGF activated the System B and System y^+ activities by generating the intracellular second messenger diacylglycerol, which activates protein kinase C. In this chapter, we used phorbol ester TPA to directly activate the cellular protein kinase C, bypassing the signal pathway between the TGF∝/EGF to the PKC.

In stimulating the System B and System y^+ activities, the onset of the TPA stimulation was slow (Fig. 7-1). Onset of activation was observed only after 8 hours, and 24 hours were needed to significantly stimulate the transport activities. Continuous TPA exposure was necessary to stimulate the transport activities.

Cycloheximide and actinomycin D blocked the TPA's stimulation effect on the System B and System y^+ activities, suggesting that a transcriptional and a translational control mechanism was involved. Whether the gene expression and the de novo protein synthesis involved the transporter protein per se, or other regulatory proteins, is not clear. Further molecular study using the cDNA probes would give a more precise answer.

Even though we did not have molecular probe for the present study, the kinetics studies of the transport activities are still useful tools. TPA increased both the system B and system y^+ transport V_{max} without changing the other kinetic parameters. The increase in V_{max}, without K_m changing, strongly suggests that increases in System B and

System y^+ activities by TPA were due to the increase in copies of functional transporter units, rather than due to modification of the transport affinity.

To further confirm that the effect of TPA was a protein kinase C activation phenomenon, we used the specific protein kinase C inhibitors chelerythrine Cl and calphostin C. As shown in the results, these PKC inhibitors blocked the TPA's effect on the System B and System y^+ activities. These data indicated that the TPA stimulation of transport activities involved protein kinase C activation, not protein kinase C inhibition.

When we compare the System B and System y^+ transport activities that were stimulated by TGF∝/EGF (chapter 6) and TPA (this chapter), we can see that each system's transporter characteristics were very similar. These data further support the notion that TGF∝/EGF stimulate the system B and system y^+ activities by engaging protein kinase C.

7.5 Summary

Phorbol ester TPA activates System B and System y^+, but not System L, activities throughout all cell ages. The activation involves transcription and translation process, and likely is mediated via protein kinase C. The phorbol ester stimulation of System B and System y^+ results in an increase in system's V_{max} without affecting the corresponding K_m.

Fig. 7-1. TPA System B stimulation time course

System B alanine (50 μM) uptake was measured in day 3 cells which had been incubated in DMEM or 1 μM TPA for various periods of time (0 - 24 hours). Continuous exposure ($\geq$ 8 hours) to TPA resulted in a increase in alanine uptake.

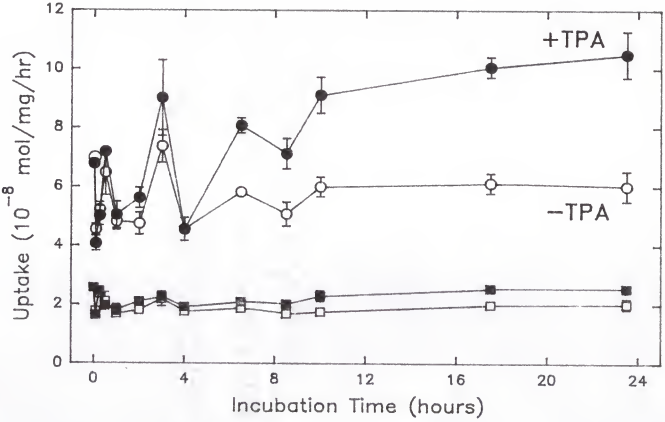

+TPA
−TPA
Uptake (10^{-8} mol/mg/hr)
Incubation Time (hours)
0
2
4
6
8
10
12
0
4
8
12
16
20
24

Fig. 7-2. Dose curve of the TPA System B stimulation

System B alanine (50 μM) uptake was measured in cells which had been incubated in various concentration of TPA (10^{-12} - 10^{-5} M) for 24 hours.

Alanine uptake, nmole mg⁻¹ min⁻¹
1.2
1.0
0.8
0.6
0.4
0.2
Na⁺–dependent
Na⁺–independent
– ∞ –12 –11 –10 –9 –8 –7 –6 –5
TPA Concentration, log[M]

Fig. 7-3. Effect of TPA on System B activity at various cell ages

System B alanine (50 μM) uptake in cells (2 days to 35 days old) which had been incubated in 0.5 μM TPA for 24 hours prior to the uptake experiments.

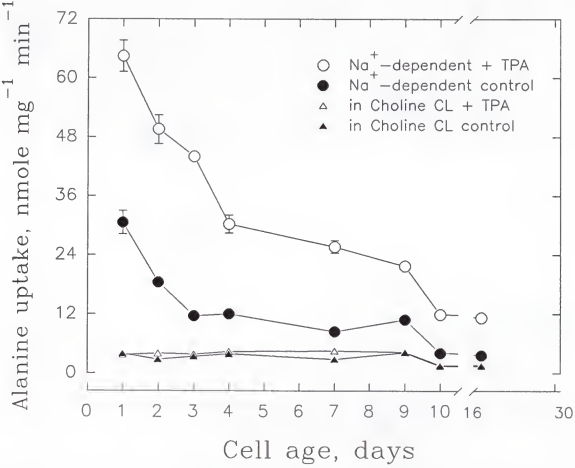

Alanine uptake, nmole mg^{-1} min^{-1}
72
60
48
36
24
12
0
0 1 2 3 4 5 6 7 8 9 10 16 30
Cell age, days
Na^+–dependent + TPA
Na^+–dependent control
in Choline CL + TPA
in Choline CL control

Fig. 7-4. The effect of CHX on the TPA-induced alanine uptake

Alanine (50 μM) uptake was measured in cells (3 days old and 9 days old) which had been incubated in 0.5 μM TPA ± 10 μM CHX for 24 hours. TPA stimulated the Na^+-dependent System B alanine uptake ($p < 0.05$, $n = 6$). CHX in the TPA incubation medium blocked this TPA's stimulation effect ($p < 0.05$, $n = 6$). The alanine uptake in choline Cl medium was not affected by TPA ($p < 0.05$, $n = 9$). Data shown were obtained from 3 days old cells, with similar results observed in 9 days old cells.

Alanine uptake, nmole mg^{-1} min^{-1}
0
0.2
0.4
0.6
0.8
in CHO
in Na
Cont
TPA
CHX
TPA+
CHX
Cont
TPA
CHX
TPA+
CHX

Fig. 7-5. The effect of actinomycin D on the TPA-induced System B activity

System B alanine (50 μM) uptake was measured in cells (3 days old) which had been incubated in 0.5 μM TPA ± 0.5 μM actinomycin D for 24 hours prior to the uptake measurements. TPA alone stimulated the System B alanine uptake ($p < 0.05$, $n = 6$), while actinomycin D in the tPA medium blocked the TPA's stimulation effect ($p < 0.05$, $n = 6$). Similar results were obtained in 9 days old cells.

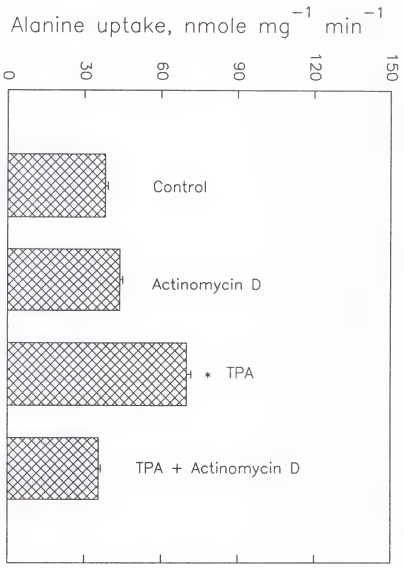

Alanine uptake, nmole mg^{-1} min^{-1}
0
30
60
90
120
150
Control
Actinomycin D
* TPA
TPA + Actinomycin D

Fig. 7-6. The effects of chelrythrine on the TPA-induced System B activity

System B alanine (50 μM) uptake was measured in day 9 cells which had been incubated 0.5 μM TPA ± 6.6 μM chelerythrine for 24 hours. The TPA stimulation of alanine uptake was blocked by the CHE ($p < 0.05$, $n = 6$).

Alanine uptake, nmole mg^{-1} min^{-1}
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Control
CHE
TPA
CHE + TPA

Fig. 7-7. The effect of TPA on System B activity kinetics

System B alanine (10 μM - 5 mM) uptake kinetics were measured in cells (2 days old and 9 days old) which had been incubated in DMEM or 0.5 μM TPA for 24 hours. System B uptake was plotted as a function of alanine concentration.

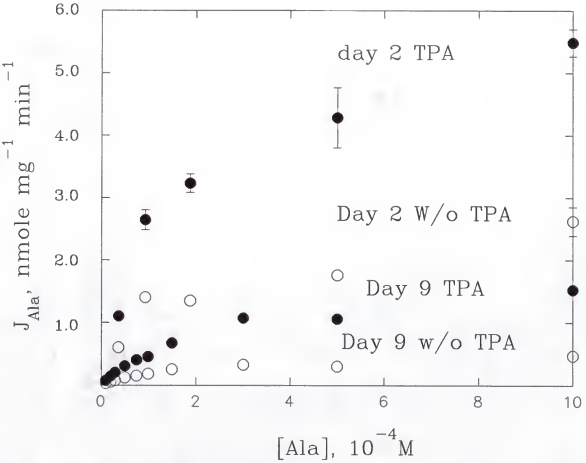

6.0
5.0
4.0
3.0
2.0
1.0
0
2
4
6
8
10
day 2 TPA
Day 2 W/o TPA
Day 9 TPA
Day 9 w/o TPA
J_{Ala}, nmole mg^{-1} min^{-1}
[Ala], 10^{-4}M

Fig. 7-8. Eadie-Hofstee transformation of TPA-induced System B activity kinetics

The kinetic data in Fig. 7-7 was re-plotted as a function of J_{ala} vs. J/[ala]. TPA increased the V_{max} in both day 2 and day 9 cells, while the K_m was not affected.

day 2 − TPA
day 2 + TPA
day 9 − TPA
day 9 + TPA
J_{ala}, nmole mg^{-1} min^{-1}
J/[Ala], μLiter mg^{-1} min^{-1}
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
0
5
10
15
20
25
30
35
40

Fig. 7-9. The effect of TPA on System y^+ activity at various cell ages

System y^+ arginine (5 μM) uptake was measured in various ages of cells which had been incubated in DMEM or DMEM plus 0.5 μM TPA for 24 hours.

Arginine uptake, pmole mg^{-1} min^{-1}
200
160
120
80
40
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Cell age, day
+ TPA
– TPA

Fig. 7-10. The effect of CHX on TPA-induced System y^+ activity

System y^+ arginine (5 μM) uptake was measured in day 2 cells which had been incubated in 0.5 μM TPA for 24 hours with or without 50 μM CHX in the first 12 hours incubation. TPA alone incubation stimulated the arginine uptake, and this stimulation was blocked by CHX in the first 12 hours TPA incubation ($p < 0.05$, $n = 3$).

Arginine uptake, pmole mg^{-1} min^{-1}
250
225
200
175
150
125
100
75
50
25
0
Control
CHX
* TPA
TPA + CHX

Fig. 7-11. The effect of chelerythrine on the TPA-induced System y^+ activity

System y^+ arginine uptake was measured in cells (9 days old) which had been incubated in DMEM, 0.5 μM TPA, 6.6 μM CHE, or 6.6 μM CHE plus 0.5 μM TPA. Arginine uptake was stimulated by TPA incubation. The TPA stimulation effect was blocked by the CHE in the TPA incubation medium ($p < 0.05$, $n = 6$). Similar results were obtained in day 2 cells.

Arginine uptake, per cent
0
50
100
150
200
250
300
Control
TPA
*
CHE
CHE + TPA

Fig. 7-12. The effect of TPA on System y^+ transport kinetics

System y^+ arginine (0.5 μM - 1 mM) uptake was measured in cells (2 days old and 9 days old) which had been incubated in DMEM or 0.7 μM TPA for 24 hours. TPA increased the V_{max} in both day 2 and day 9 cells without affecting the k_m.

day 2 + TPA
day 2 − TPA
day 9 + TPA
day 9 − TPA
J_{Arg}, pmole mg^{-1} min^{-1}
$J_{org}/[Arg]$, μLiter mg^{-1} min^{-1}
0
100
200
300
400
500
600
700
800
4
8
12
16
20

CHAPTER 8
SUMMARY AND CONCLUSIONS

8.1 SUMMARY

We have studied amino acid transport systems independently serve alanine or arginine in the apical membrane of the human intestinal epithelial cell line, Caco-2. We have investigated the in vitro cellular mechanisms that underlie the independent regulation of these systems.

The structurally different substrates L-alanine and L-arginine are transported separately by unique transport pathways. The pathways serving alanine are: System B, System L, and simple passive diffusion. The pathways for arginine are System y^+ and simple passive diffusion. The transport activities of System B, System y^+, and System L were each-down regulated in parallel with advancing Caco-2 cell development and differentiation. System B and System y^+ activities were each actually trans-stimulated by their own substrate. System B capacity was chronically up-regulated by its own substrates through a mechanism that involved protein kinase C and de novo protein synthesis. Simple passive diffusion of each substrate was unaffected by any cellular conditions that affected the carrier-mediated transport.

The peptide growth factors EGF and TGFα stimulated System

B and System y^+ transport activities. A wide variety of other growth factors and hormones were without effect. The relative transport capacities of Systems B and y^+ paralleled the activation or inhibition of protein kinase C. Furthermore, inhibition of protein kinase C or inhibition of protein synthesis each prevented the EGF/TGFα activation of Systems B or y^+.

In addition to the biochemical and histological similarities of Caco-2 cells and normal small intestinal enterocytes, the Caco-2 apical membrane also possesses the same alanine and arginine transport systems found in the small intestinal epithelial brush border membrane. The transport activities changes that occur during Caco-2 cell development also resemble those found in the small intestinal cells. The independent regulation of the stated alanine and arginine transporters by their transported substrates, was strikingly consistent with that measured in vivo.

This study provides a better understanding of the mechanism of small intestinal nutrient absorption, provides information concerning enterocytic development, and helps our understanding of the fundamentals physiology of epithelial transporter regulation. This project provides an excellent in vitro model for future studies of intestinal regulation of nutrient absorption in states of health and disease, including adenocarcinoma development.

8.2 Conclusions

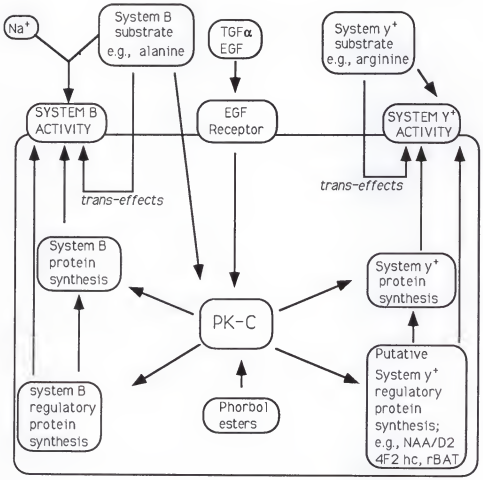
(1) Caco-2 epithelial differentiation status is associated with regulation of amino acid transport.

(2) Amino acid transporters System B and System y^+ independently serve alanine and arginine, and are regulated independently.

(3) Amino acid substrates up-regulate their own transporters' activities via trans-stimulation, or chronically by a mechanism involving de novo protein synthesis.

(4) Chronic up-regulation of System B or System y^+ capacities by de novo protein synthesis is activated by EGF/TGFα acting through a protein kinase C pathway.

The Proposed Amino Acid Transport Regulation Mechanism in Caco-2 Cells:



Amino Acid Transport Regulation in Caco-2 Cells

REFERENCE LIST

Akiyama, T., Saito, T., Ogawara, H., Toyoshima, K. and Yamamoto, T. 1988. Tumor promoter and epidermal growth factor stimulate phosphorylation of the c-erbB-2 gene production in MKN-7 human adenocarcinoma cells. Mol. Cell. Biol 8:1019-1026

Betran, J., Werner, A., Moore, M.L., Stange, G., Markovich, D. 1992. Expression cloning of a cDNA from rabbit kidney cortex that induces a single transport system for cystine and dibasic and neutral amino acids. Proc. Natl. Acad. Sci. USA. 89:5601-5

Bruns, R. F, Smith, D. R, and Nakono, K. 1991. Inhibition of protein kinase C by calphostin C is light-dependent. Biochem. Biophy. Res. Comm. 176:288-293

Buddington, R.K., Chen, J.W. Diamond, J.M. 1987. Genetic and phenotypic adaptation of intestinal nutrient transport to diet in fish. J. Physiol. Lond. 393: 261-81

Buddington, R.K., Chen, J.W., Diamond, J.M. 1991. Dietary regulation of intestinal brush-border sugar and amino acid transport in carnivores. Am. J. Physiol. 261(4 Pt 2): R793-801

Buddington, R.K., Diamond, J.M. 1987. Pyloric ceca of fish: a "new" absorptive organ. Am. J. Physiol. 252(1 Pt 1): G65-76

Buddington, R.K., Diamond, J.M. 1989. Ontogenetic development of intestinal nutrient transporters. Annu. Rev. Physiol. 51: 601-19

Buddington, R.K., Diamond, J.M. 1990. Ontogenetic development of monosaccharide and amino acid transporters in rabbit intestine. Am. J. Physiol. 259(4 Pt 1): G544-55

Campa, M.J., Kilberg, M.S. 1989. Characterization of neutral and cationic amino acid transport in Xenopus oocytes. J. Cell. Physiol.141(3): 645-52

Carpenter, G., King, L. Jr., Cohen, S. 1979. Rapid enhancement of protein phosphorylation in A-431 cell membrane preparations by epidermal growth factor. J. Biol. Chem 254:4884-4891

Carpenter, G., Wahl, M.I. 1990. The epidermal growth factor family. In: Peptide growth factors and their receptors I, edited by Sporn, M.B. and Roberts, A.B, SpringerVerlag, Germany. pp69-172

Carver, J. A, Cooke, R. M., Esposito, G., Campbell, I. D., Gregory, H., and Sheard, B. 1986. A high resolution H NMR study of the solution structure of human epidermal growth factor, FEBS Lett 205:77-81.

Chen, W.S., Lazar, C.S., Poenie, M., Tsien, R.Y., Gill, G.N., Rosenfeld, M.G. 1987. Requirement for intrinsic protein tyrosine kinase in the immediate and late actions of the EGF receptor. Nature. Aug 27-Sep 2; 328(6133): 820-3

Christensen, H.N. 1984. Organic ion transport during seven decades. Biochim. Biophys. Acta 779:225-69

Christensen, H.N. 1985. On the strategy of kinetic discrimination of amino acid transport systems. J. Membr. Biol. 84:97-103

Christensen, H.N. 1990. Role of amino acid transport and countertransport in nutrition and metabolism. Physiol. Rev.70:43-77

Christensen, H.N., Riggs, T.R., Fischer, H., Palatine, I.M. 1952. Amino acid concentration by a free cell neoplasm: Relations among amino acids. J. Biol. Chem. 198:1-22

Christensen, H.N., Oxender, D.L., Liang, M., Vatz. K.A. 1965. The use of N-methylation to direct the route of mediated transport of amino acids. J. Biol. Chem. 240:3609-16.

Christensen, H.N., Liang, M. 1966. Transport of diamino acids into the Enrlich cells. J. Biol. Chem. 241:5542-51

Dawson, W.D., Cook, J.S. 1987. Parrallel changes in amino acid transport and protein kinase C localization in LLC-PK1 cells treated with TPA or diradylglycerols. J. Cell. Physiol. 132:104

Diamond, J.M. 1991. Evolutionary design of intestinal nutrient absorption:enough but not too much. NIPS. 6:92-96

Diamond, J.M., Karasov, W.H. 1987. Adaptive regulation of intestinal nutrient transporters. Proc. Natl. Acad. Sci. U. S. A. 84(8): 2242-5

Edelman, A.M., Blumenthal, D.K., Krebs, E.G. 1987. Protein serine/threonine kinases. Annu. Rev. Biochem. 56: 567-613

Ellis, C., Moran, M., McCormick, F., and Pawson, T. 1990. EGF stimulates GAP in vitro. Nature 311:377-381.

Englesberg, E., Moffett, J., Perier, F. 1986. Mechanism of regulation of amino acid transport in CHO-K1 cells and the insulin connection. Federation Pro. 45(10):2441-2443

Fava, R. A. and Cohen, S. 1984. Isolation of a calcium-dependent 35 kilodalton substrate for the EGF receptor/kinase from A-431 cells. L. Biol. Chem 258:2636-2645

Ferraris, R.P., Diamond, J., Kwan, W.W. 1988a. Dietary regulation of intestinal transport of the dipeptide carnosine. Am. J. Physiol. 255(2 Pt 1): G143-50

Ferraris, R.P., Kwan, W.W., Diamond, J. 1988b. Regulatory signals for intestinal amino acid transporters and peptidases. Am. J. Physiol. 255(2 Pt 1): G151-7

Ferraris, R.P., Diamond, J.M. 1989. Specific regulation of intestinal nutrient transporters by their dietary substrates. Annu. Rev. Physiol. 51: 125-41

Frexes-Steed, M., Warner, M.L., Bulus, N., Flakoll, P. 1990. Abumrad-NN. Role of insulin and branched-chain amino acids in regulating protein metabolism during fasting. Am. J. Physiol. 258(6 Pt 1): E907-17

Gaull, G.E., Wright, C.E., Isaacs, C.E. 1985. Significance of growth modulators in human milk. Pediatrics. 75(1 Pt 2): 142-5

Gerencser, G.A., Stevens, B.R. 1989. Energetics of sodium-coupled active transport mechanisms in invertebrate epithelia. Am. J. Physiol. 257(3 Pt 2): R461-72

Glenney, J.R. Jr., Chen, W.S., Lazar, C.S., Walton, G.M., Zokas, L.M., Rosenfeld, M.G., Gill, G.N. 1988. Ligand-induced endocytosis of the EGF receptor is blocked by mutational inactivation and by microinjection of anti-phosphotyrosine antibodies. Cell. 52(5): 675-84

Grasset, E., Pinto, M., Dussaulx, E., Zweibaum, A., Desjeux, J.F. 1984. Epithelial properties of human colonic carcinoma cell line Caco-2: electrical parameters. Am. J. Physiol. 247(3 Pt 1): C260-7

Grasset, E., Bernabeu, J., Pinto, M. 1985. Epithelial properties of human colonic carcinoma cell line Caco-2: effect of secretagogues. Am. J. Physiol. 248(5 Pt 1): C410-8

Herbert, J. M, Smith, D. B, and Satoma, G. 1990. Chelerythrine is a potent and specific inhibitor of protein kinase C. Bichem. Biophys. Res. Comm. 172:993-999.

Hernandez-Sotomayor, S.M.T., Carpenter, G. 1992. Epidermal growth factor receptor:elements of intracellular communication. J. Memb. Biol. 128:81-89.

Hidalgo, I.J., Kato, A., Borchardt. 1989. Binding of epidermal growth factor by human colon carcinoma cell (Caco-2) monolayers. Biochim. Biophy. Res. Comm. 160(1):317-324

Honegger, A.M., Dull, T.J., Felder, S., Van Obberghen, E., Bellot, F., Szapary, D., Schmidt, A., Ullrich, A., Schlessinger, J. 1987a. Point mutation at the ATP binding site of EGF receptor abolishes protein-tyrosine kinase activity and alters cellular routing. Cell. 51(2): 199-209

Honegger, A.M., Szapary, D., Schmidt, A., Lyall, R., Van Obberghen, E., Dull, T.J., Ullrich, A., Schlessinger, J.A. 1987b. Mutant epidermal growth factor receptor with defective protein tyrosine kinase is unable to stimulate proto-oncogene expression and DNA synthesis. Mol. Cell. Biol. 7(12): 4568-71

Hopfer, U. 1987. Membrane transport mechanism for hexoses and amino acids in the small intestine. In: Physiology of the Gastrointestinal Tract, edited by Johnson-LR. Raven Press, New York. pp 1499-1526

Jumarie, C., Malo, C. 1991. Caco-2 cells cultured in serum-free medium as a model for the study of enterocytic differentiation in vitro. J. Cell. Physiol. 149(1): 24-33

Karasov, W.H., Diamond, J.M. 1983. Adaptive regulation of sugar and amino acid transport by vertebrate intestine. Am. J. Physiol. 245(4): G443-62

Karasov, W., Solberg, D., Carter, S., Hughes, M., Phan, D., Zollman, F., Diamond, J. 1986. Uptake pathways for amino acids in mouse intestine. Am. J. Physiol. 251(4 Pt 1): G501-8

Karasov, W.H., Solberg, D.H., Diamond, J.M. 1987. Dependence of intestinal amino acid uptake on dietary protein or amino acid levels. Am. J. Physiol. 252(5 Pt 1): G614-25

Kilberg, M.S. 1986. Amino acid transport in eukaryotic cells and tissues. Federation Pro. 45(10): 2438-2440

Kilberg, M.S., Handlogten, M.E., Christensen, H.N. 1981. Characteristics of system ASC for transport of neutral amino acids in the isolated rat hepatocyte. J. Biol. Chem. 256(7): 3304-12

Kilberg, M.S., Bracy, D.S., Handlogten, M.E. 1986. Substrate regulation of hepatic system A transport activity after induction by substrate starvation or glucagon. Fed. Proc. 45(10): 2438-41

Kilberg, M.S, Stevens, B.R., Novak, D.A. 1993. Recent advances in mammalian amino acid transport. Annu. Rev. Nutr. 13:137-65

Kim, J.W., Closs, E.I., Albritton, L.M., Cunningham, J.M. 1991. Transport of cationic amino acids by the mouse ecotropic retrovirus receptor [see comments]. Nature. 352(6337): 725-8

Klip, A., Douen, A.G. 1989. Role of kinases in insulin stimulation of glucose transport. J. Membr. Biol. 111(1): 1-23

Koboyashi, E, Mituta, M, and Suzuki, H. 1989. Calphostin C, a novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. Bichem. Biophy. Res. Comm. 159:548-553

Koyama, S.Y., Podolsky, D.K. 1989. Differential expression of transforming growth factors alpha and beta in rat intestinal epithelial cells. J. Clin. Invest. 83(5): 1768-73

Laganiere, S., Maestracci, D., Berteloot, A. 1986. Adaptation of intestinal sugar and amino acid transport to gastric hyperalimentation. Clin. Invest. Med. 176-85

Magagnin, S., Bertran, J., Werner, A., Markovich, D., Biber, J., Palacin, M., Murer, H. 1992. Poly(A)+ RNA from rabbit intestinal mucosa induces b0,+ and y+ amino acid transport activities in Xenopus laevis oocytes. J. Biol. Chem. 267(22): 15384-90

Malo, C. 1991. Multiple pathways for amino acid transport in brush border membrane vesicles isolated from the human fetal small intestine. Gastroenterology. 100(6): 1644-52

Margolis, B. L., Rhee, S. G., Felder, S. Mervic, M., Lyall R., Levitzki, A., Zilberstein, A., and Schlessinger. 1989. EGF induces tyrosine phosphorylation of phospholipase C-II: A potential mechanism for EGF receptor signaling. Cell 57:1101-1107

Mayo, K.H., Cavalli, R.C., Peters, A.R., Boelens, R., Kaptein, R. 1989. Sequence-specific 1H-n.m.r. assignments and peptide backbone conformation in rat epidermal growth factor. Biochem. J. 257(1): 197-205

Meisenhelder, J., Suh, P. G., Rhee, S. G., Hunter, T. 1989. Phospholipase C-y is a substrate for the PDGF and EGF receptor protein-kinases in vivo and in vitro. Cell 57:1109-1122

Mircheff, A.K., Sachs, G., Hanna, S.D., Labiner, C.S., Rabon, E., Douglas, A.P., Walling, M.W., Wright, E.M. 1979. Highly purified basal lateral plasma membranes from rat duodenum. Physical criteria for purity. J. Membr. Biol. 50(3-4): 343-63

Mircheff, A.K., van Os, C.H., Wright, E.M. 1980. Pathways for alanine transport in intestinal basal lateral membrane vesicles. J. Membr. Biol. 52(1): 83-92

Molloy, C. J., Bottaro, D. P., Fleming, T. P., Marshall, M. S., Gibbs, J. B., Aaronson, S. A. 1990. GAP is a possible epidermal growth factor receptor substrate. Nature 342:711-714

Montelione, G.T., Winkler, M.E., Burton, L.E., Rinderknecht, E., Sporn, M.B., Wagner, G. 1989. The solution sequence-specific 1H-NMR assignments and identification of two small antiparallel beta-sheets in structure of recombinant human transforming growth factor alpha. Proc. Natl. Acad. Sci. USA. 86(5): 1519-23

Moolenaar, W. H., Tertoolen, L. G. J., de Laat, S. W. 1984. Rapid increase of intracellular DAG level in A-431 cells. J. Biol. Chem. 259:8060-8069

Moolenaar, W. H., Bierman, A. J., Tilly, B. C., Verlaan, I., Defize, L. H., Ullrich, A., and Schelessinger, J. 1988. A point mutation at the ATP-binding site of the EGF receptor abolishes signal transduction. EMBO J. 8:707-710

Munck, B.G. 1981. Intestinal absorption of amino acids. In: Physiology of the Gastrointestinal Tract, edited by Johnson-LR. Raven Press, New York. pp 1097-1122

Oxender, D.L. and Christensen, H.N. 1963. Distinct mediating systems for the transport of neutral amino acids ny the Ehrlich cell. J. Biol. Chem. 238:3686-99

Pan, M., Neu, J., Stevens, B.R. 1991. Regulation of alanine transport in human intestinal epithelial cell line Caco-2. FASEB J. 5(4):A760

Ray, P., Moy, F.J., Montelione, G.T., Liu, J.F., Narang, S.A., Scheraga, H.A., Wu, R. 1988. Structure-function studies of murine epidermal growth factor: expression and site-directed mutagenesis of epidermal growth factor gene. Biochemistry. 27(19): 7289-95

Reiser, S. and Christiansen, P.A. 1971a. The properties of the preferential uptake of L-leucine by isolated intestinal epithelial cells. Biochim. Biophys. Acta. 225(1): 123-39

Reiser, S. and Christiansen, P.A. 1971b. Inhibition of amino acid uptake by ATP in isolated intestinal epithelial cells. Biochim. Biophys. Acta. 233(2): 480-4

Reiser, S. and Christiansen, P.A. 1971c. Stimulation of basic amino acid uptake by certain neutral amino acids in isolated intestinal epithelial cells. Biochim. Biophys. Acta. 241(1): 102-13

Rousset, M., Laburthe, M., Pinto, M, Chevalier, G., Rouyer-Fessard, C., Dussaulx, E., Trugnan, G., Boige, N., Brun, J.L., Zweibaum, A. 1985. Enterocytic differentiation and glucose utilization in the human colon tumor cell line Caco-2: modulation by forskolin. J. Cell. Physiol. 123(3): 377-85

Saier, M.H. Jr., Daniels, G.A., Boerner, P., Lin, J. 1988. Neutral amino acid transport systems in animal cells: potential targets of oncogene action and regulators of cellular growth. J. Membr. Biol. 104(1): 1-20

Salloum, R.M., Souba, W.W., Fernandez, A., Stevens, B.R. 1990. Dietary modulation of small intestinal glutamine transport in intestinal brush border membrane vesicles of rats. J. Surg. Res. 48(6): 635-8

Salloum, R.M., Stevens, B.R., Souba, W.W. 1991. Adaptive regulation of brush-border amino acid transport in a chronic excluded jejunal limb. Am. J. Physiol. 261(1 Pt 1): G22-7

Souba, W.W., Klimberg, V.S., Plumley, D.A., Salloum, R.M, Flynn, T.C., Bland, K.I., Copeland, E.M. 3d. 1990. The role of glutamine in maintaining a healthy gut and supporting the metabolic response to injury and infection. J. Surg. Res. 383-91

Souba, W. W., Pan, M., and Stevens, B. R. 1992. Kinetics of the Sodium-dependent glutamine transporter in human intestinal cell confluent monolayers. Bioch. Biophy. Res. Comm. 188(2):746-753

Stein, E.D., Chang, S.D., Diamond, J.M. 1987. Comparison of different dietary amino acids as inducers of intestinal amino acid transport. Am. J. Physiol. 252(5 Pt 1): G626-35

Stelzner, M., Buddington, R.K., Phillips, J.D., Diamond, J.M., Fonkalsrud, E.W. 1990a. Changes in mucosal nutrient transport in small and large ileal reservoirs after endorectal ileal pullthrough. J. Surg. Res. 49(4): 344-55

Stelzner, M., Fonkalsrud, E.W., Buddington, R.K., Phillips, J.D., Diamond, J.M. 1990b. Adaptive changes in ileal mucosal nutrient transport following colectomy and endorectal ileal pull-through with ileal reservoir. Arch. Surg. 125(5): 586-90

Stern, D. F. and Kemps, M. P. 1988. EGF-stimulated tyrosine phosphorylation of $p185^{neu}$: a potential model for receptor interactions. EMBO J. 7:995-1001

Stevens, B.R. Amino acid transport in intestine. 1992a. In: Mammalian Amino Acid Transport, edited by Kilberg, M.S. and Haussinger, D., Plenum Press, New York, 149-163

Stevens, B.R. 1992b. Vertebrate intestine apical membrane mechanisms of organic nutrient transport. Am. J. Physiol. 263:R458-63

Stevens, B.R., Ross, H.J., Wright, E.M. 1982. Multiple transport pathways for neutral amino acids in rabbit jejunal brush border vesicles. J. Membr. Biol. 66(3): 213-25

Stevens, B.R., Kaunitz, J.D., Wright, E.M. 1984. Intestinal transport of amino acids and sugars: advances using membrane vesicles. Annu. Rev. Physiol. 46: 417-33

Stevens, B.R. and Wright, E.M. 1985. Kinetic model of the brush-border proline/sodium (IMINO) cotransporter. Ann. N. Y. Acad. Sci. 456: 115-7

Stevens, B.R., Kempner, E.S., Wright, E.M. 1986. Radiation inactivation probe of membrane-bound enzymes: gamma-glutamyltranspeptidase, aminopeptidase N, and sucrase. Anal. Biochem. 158(2): 278-82

Stevens, B.R., Wright, E.M. 1987. Kinetics of the intestinal brush border proline (Imino) carrier. J. Biol. Chem. 262(14): 6546-51

Tate, S.S., Yan, N.N., Udenfriend, S. 1992. Expression cloning of a Na(+)-independent neutral amino acid transporter from rat kidney. Proc. Natl. Acad. Sci. USA. 89(1): 1-5

Van Winkle, L.J. 1988. Amino acid transport in developing animal oocytes and early conceptuses. Biochim. Biophys. Acta. 947(1): 173-208

Van Winkle, L.J., Campione, A.L., Gorman, J.M. 1988. Na+-independent transport of basic and zwitterionic amino acids in mouse blastocysts by a shared system and by processes which distinguish between these substrates. J. Biol. Chem. 263(7): 3150-63

Van Winkle, L.J. and Campione, A.L. 1990. Functional changes in cation-preferring amino acid transport during development of preimplantation mouse conceptuses. Biochim. Biophys. Acta. 1028(2): 165-73

Van Winkle, L.J., Campione, A.L., Gorman, J.M., Weimer, B.D. 1990. Changes in the activities of amino acid transport systems b0,+ and L during development of preimplantation mouse conceptuses. Biochim. Biophys. Acta. 1021(1): 77-98

Wahl, M. I., Nishibe, S., Pann-Ghill S., Rhee, S. G., Carpenter, G. 1989. Epidermal growth factor stimulates tyrosine phosphorylation of phospholipase c-II independently of receptor internalization and extracellular calcium. Proc. Natl. Acad. Sci. USA. 86:1568-1572

Wang, H., Kavanaugh, M.P., North, R.A., Kabat, D. 1991. Cell-surface receptor for ecotropic murine retroviruses is a basic amino-acid transporter [see comments]. Nature. 352(6337): 729-31

Wells, R.G., Hediger, M.A. 1992a. Cloning of a rat kidney cDNA that stimulates dibasic and neutral amino acid transport and has sequence similarity to glucosidases. Proc. Natl. Acad. Sci. USA. 89(12): 5596-600

Wells, R.G. Lee, W.S., Kanai, Y., Leiden, J.M., Hediger, M.A. 1992b. The 4F2 antigen heavy chain induces uptake of neutral and dibasic amino acids in Xenopus oocytes. J. Biol. Chem. 267(22): 15285-8

White, M. F. 1985. The transport of cationic amino acids across the plasma membrane of mamalian cells. Biochem. Biophys. Acta. 822:355-74

Whittman, M., Downes, C. P., Keeler, T., Cantley, L. 1988. Epidermal growth factor receptor stimulates Pi-s kinase. Nature 332:644-647

Wice, B.M., Trugnan, G., Pinto, M., Rousset, M., Chevalier, G., Dussaulx, E., Lacroix, B., Zweibaum, A. 1985. The intracellular accumulation of UDP-N-acetylhexosamines is concomitant with the inability of human colon cancer cells to differentiate. J. Biol. Chem. 260(1): 139-46

Zweibaum, A., Triadou, N., Kedinger, M., Augeron, C., Robine, R., Leon, S., Pinto, M., Rousset, M., Haffen, K. 1983. Sucrase-isomaltase: a marker of foetal and malignant epithelial cells of the human colon. Int. J. Cancer. 32(4): 407-12

BIOGRAPHICAL SKETCH

Ming Pan, born on February 5, 1963 in ShaoGuan City of the GuangDong Province, People's Republic of China. His father is Jiaan Pan, mother is FengQun Chen. He went to elementary and middle schools in ShaoGuan city. In 1980, after graduating from high school, he went to Sun Yat-Sen University of Medical Sciences studying medicine, specializing in surgery. He graduated from medical school with a Medical Bachelor (M.B) degree in 1986. He then worked in the YueBei People's Hospital in ShaoGuan as a surgeon during 1986-1987. He came to United States of America in 1987. He entered the Ph.D program in the Department of physiology, College of Medicine, University of Florida in May 1988. Under the guidance of Dr. Bruce R. Steven, he has been studying the amino acid transport regulation in the small intestine. He has completed all the degree requirements and successfully defended his dissertation. He is expected to graduate and receive a Doctor of Philosophy degree in Physiology in May 1993.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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May, 1993

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